JANUARY-FEBRUARY 1954

The Journal of Infectious Diseases

Published by the John Appleton McCormick Memorial Institute for Infectious Diseases

under the Editorship of LUDVIC HEKTOEN and EDWIN O. JORDAN

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PUBLISHED FOR THE JOHN ROCKEFELLER McCORMICK MEMORIAL

OF THE UNIVERSITY OF CHICAGO

THE UNIVERSITY OF CHICAGO PRESS

CHICAGO · ILLINOIS · U.S.A.

The Journal of Infectious Diseases

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This journal was established in connection with the John Rockefeller McCormick Memorial Institute for Infectious Diseases, by the munificence of the late Mrs. Edith Rockefeller McCormick and the late Mr. Harold F. McCormick. Since 1[illegible] it has been published in connection with the John Rockefeller McCormick Memorial Fund of the University of Chicago.

The Journal of Infectious Diseases is published bi-monthly by The University of Chicago Press, 5750 Ellis Avenue, Chicago, Illinois. The subscription price is $7.00 per year; $3.50 a volume; the price of single copies of current volume is $2.00. ¶ Postage is prepaid by the publishers on all orders from the United States, Mexico, Cuba, Puerto Rico, Panama Canal Zone, Republic of Panama, Dominican Republic, Canary Islands, El Salvador, Argentina, Bolivia, Brazil, Colombia, Chile, Costa Rica, Ecuador, Guatemala, Honduras, Nicaragua, Peru, Haiti, Uruguay, Paraguay, Hawaiian Islands, Balearic Islands, Spain and Venezuela. ¶ Postage is charged extra as follows: for Canada and Newfoundland, 40 cents on annual subscriptions (total $7.40); on single copies 10 cents (total $2.10); for all other countries in the Postal Union, 75 cents on annual subscriptions (total $7.75); on single copies 15 cents (total $2.15). ¶ Patrons are requested to make all remittances payable to the University Press in postal or express money orders or bank drafts.

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Business correspondence should be addressed to The University of Chicago Press, 5750 Ellis Avenue, Chicago 37, Illinois.

Communications for the editors and manuscripts should be addressed to Mrs. Dorothy W. Green, Assistant to the Editor, The Journal of Infectious Diseases, 5750 Ellis Avenue, Chicago 37, Illinois.

Entered as second-class matter August 1, 1911, at the post office at Chicago, Illinois, under the act of March 3, 1879.

Acceptance for mailing at special rate of postage provided for in Digest of Postal Laws and Regulations, Section 1103, Act of October 3, 1917, as amended February 28, 1925, authorized June 5, 1928.

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J. Infect. Dis. Vol. 94, No. 1

A DISEASE IN CAPTIVE EGRETS CAUSED BY A VIRUS OF THE PSITTACOSIS-LYMPHOGRANULOMA VENEREUM GROUP

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In a previous publication, Rubin et al[1] reported the isolation of a virus of the psittacosis-lymphogranuloma venereum group from the blood of two wild snowy egrets taken in southeastern Louisiana. The similarity in experimental host range of this agent to that of Louisiana pneumonitis (Borg strain), which was responsible for a severe human pneumonitis outbreak in 1943,[2] was noted, and attention was called to the role the newly isolated agent might play in the epidemiology of human respiratory infection of unknown origin. The need to clarify the relationship of the egret virus to agents known to be pathogenic for humans has been further accentuated by the finding of this agent among another group of birds captured a year after the first egret isolation in Louisiana. This paper deals with these isolations and with some additional work done to characterize the agents isolated from both groups.

History

On June 19, 1951, 19 young American egrets and 23 young snowy egrets were captured† in the southeastern part of Louisiana and brought to the laboratory at Montgomery, Alabama. Here they were housed in large outdoor cages, and transferred indoors for use in various studies on the epidemiology of eastern equine encephalomyelitis then in progress at the laboratory.

On August 15th, one of the American egrets in an outdoor cage was observed to be emaciated and extremely weak, and was found dead later that day. In the ensuing two months, 18 American egrets, the entire laboratory population, died. Death usually occurred within 1 or 2 days of the sudden manifestations of ruffling of the feathers, immobility, weakness, and floundering. On September 5, 1951, in the midst of the American egret epizootic, a snowy egret in an adjoining cage died, initiating a similar epizootic among the snowy egrets.

Gross pathological findings

Autopsy revealed that the birds had lost a great deal of weight, and there was wasting of the pectoral muscles. The spleen and liver were swollen, and the latter was usually pale, with areas of infarction occasionally present. A fibrinous exudate frequently was seen in the peritoneal cavity and the air sacs were thickened.

Isolation of the agent

The epidemiology and pathology of the disease indicated that it was contagious and systemic in nature and caused by a single infectious entity. Attempts to culture a bacterial organism were unsuccessful. Smears made of the spleens and livers and stained

Received for publication April 15, 1953.

* Present address: Biology Division, California Institute of Technology, Pasadena, California.

† Captured by Dr. Robert Kissling and Dr. Roy Chamberlain.

1. Rubin, H., Kissling, H. E., Chamberlain, R. W., Eidson, M. E. 1951, Proc. Soc. Exper. Biol. & Med. **78**: 696–698.
2. Olson, B. J. and Treuting, W. L. 1944, Pub. Health Rep. **60**: 1488–1503.

with Giemsa's stain revealed minute coccoid bodies, resembling elementary bodies of the psittacosis-lymphogranuloma venereum group.

Some of the later cases were complicated by the fact that the birds had been used in successful transmission studies with eastern equine encephalomyelitis (E.E.E.), experiencing a transient viremia with this agent. In most of these birds, however, death did not occur until 1 week to 1 month after the viremia, and the pathology was identical with that seen in the early cases where there had been no exposure to the E.E.E. virus. Two of the birds, however, died within a week of the transient viremia. These two presented no gross pathology, and E.E.E. was recovered from their brains. The other cases were clearly not due to the E.E.E. agent since the pathology was distinctly different and no virus could be recovered from the central nervous system.

In order to isolate the responsible agent, suspensions were made of the liver and the spleen, and, in some instances, the heart, lung, kidney, intestine, and brain. These were inoculated intracerebrally and intraperitoneally into different groups of mice, five for each organ suspension and for each route of inoculation.

All mice inoculated intraperitoneally with either liver or spleen suspensions from the second bird to succumb died 7 to 10 days after inoculation. The liver and spleen from one of these mice were smeared on glass slides and Giemsa's stain was applied. Microscopic observation revealed clusters of minute blue-staining coccoid bodies similar to those seen in the birds' organs and typical of the psittacosis group of viruses. Thereafter, the brains, livers, and spleens of inoculated mice exhibiting signs of infection were routinely examined for the presence of psittacosis-like elementary bodies.

Tissue suspensions were made from ten American egrets and two snowy egrets and inoculated into mice. The agent was first confirmed as a member of the psittacosis-lymphogranuloma venereum group by complement fixation with serums from animals immunized against a classical psittacosis strain.

In order to further identify the agent and its relation to other members of the psittacosis group, passage was continued of material from two of the American egrets and one of the snowy egrets. It was, of course, important to know the relation of this agent to the virus isolated from two noncaptive snowy egrets in 1950,[1] and to other members of the psittacosis-lymphogranuloma group of viruses. Particular attention was paid to the infectivity of the material for several species by different routes of inoculation, and the prophylactic effects of different antibiotics, since there is no uniformly reliable serological method of differentiating viruses of the psittacosis-lymphogranuloma venereum group.[3] An attempt was made, however, to detect distinguishing serological properties of the agent, using the toxin and intranasal infectivity neutralization, but no clear-cut results were obtained. Finally, to learn as much as possible about the distribution of the agent in nature, all the wild bird serums which were available in the laboratory and not earmarked for other uses were tested for antibodies to a known psittacosis antigen by both the direct and indirect, or inhibitory, complement fixation techniques.

Throughout the rest of this paper, the most recently isolated agent will be referred to as egret strain 1951, and

3. Meyer, K. F. and Eddie, B. 1952, Archiv. für die Gesamte Virus Forschung, R. Doerr and C. Hallauer, pp. 579–590.

that strain reported earlier[1] as egret strain 1950. Other strains employed in these studies were the 6 BC standard laboratory strain of psittacosis, and the Borg strain of Louisiana pneumonitis isolated from a severe human epidemic in 1943,[2] both supplied by Dr. Dorland Davis of the National Institutes of Health.

Complement fixation

The finding of elementary bodies should be supported by immunological evidence before a pathogen can be established as a member of the psittacosis-lymphogranuloma venereum group. Complement fixation is most useful for this purpose, since the various agents of the group exhibit great uniformity in this test.

The egret strain 1950 has previously been shown to fix complement with a known positive antipsittacosis serum, and to induce the formation of antibodies characteristic of the psittacosis group.[1]

The 1951 strain was likewise demonstrated to be an effective psittacosis antigen, eliciting antibody formation in vaccinated guinea pigs, and fixing complement with antiserums to standard strains of psittacosis.

As was expected, no immunological difference between the 6 BC, Borg and egret strains could be demonstrated by this technique.

Chickens were also immunized against the four strains, and their serums tested with a commercial psittacosis antigen by the direct and indirect or inhibitory complement fixation techniques. It was felt that using chick embryo material to immunize adult chickens would decrease the response to the normal tissue proteins, and would result in a more specific test for the different viruses used. However, this did not prove to be so. The serums from the immunized chickens were not able to fix complement at all in the direct test, and there was complete cross reaction between all strains in the indirect test. The results of the indirect test are presented in table 1.

Pathogenicity

Chick embryos.—After three serial mouse passages, a passage was made into the yolk sac of 6-day-old chick embryos. Death of the embryo occurred in 3 to 5 days, and Giemsa-stained smears of the yolk sacs demonstrated great numbers of elementary bodies typical of psittacosis-like viruses.

Suspensions made from the infected yolk sacs homogenized in their own yolk proved infectious in much higher dilutions than did the straight mouse passage material, and yolk sac material was therefore used in all the work to be reported except where otherwise stated.

Mice.—Early mouse brain, or liver and spleen passages of the 1951 egret strain produced death in 3 to 8 days on

TABLE 1.—*Indirect complement fixation test with psittacosis antigen and serums of chickens immunized with specified agents.*

Serum	Indirect or inhibitory serum dilution					
	1:4	1:8	1:16	1:32	1:64	1:128
Anti-Borg	−	±	++	+++	++++	++++
Anti-6 BC	−	−	−	+	+	++++
Anti-egret 1950	−	±	±	++	++	++
Anti-egret 1951	−	−	−	±	++	++++

−, Complete inhibition of complement fixation (indicates a positive reaction).
+, 75% inhibition of complement fixation.
++, 50% inhibition of complement fixation.
+++, 25% inhibition of complement fixation.
++++, No inhibition of complement fixation (indicates a negative reaction).

further intracerebral inoculation into mice, depending on the concentration employed. Death in intraperitoneally inoculated mice usually occurred 2 to 3 days later. The LD_{50} titer of this mouse material was $10^{-4.0}$ by the intracerebral route and $10^{-3.5}$ by the intraperitoneal route.

Since the most distinctive characteristic of the 1950 egret strain and the Borg strain was their ability to cause severe infection and death by any route of inoculation in mice and guinea pigs, some comparisons were made of the virulence of the various strains in our possession. Serial tenfold dilutions were made in saline and were inoculated by the intracerebral, intraperitoneal, intramuscular, and subcutaneous routes into mice. The results of the mouse titration are presented in table 2, where the logarithm of the reciprocal of the dilution calculated to be one LD_{50} according to the Reed and Muench formula[4] gives some indication of the relative infectiousness of a strain by a given route.

It can be readily seen that the 1950 and 1951 egret strains and the Borg strain are similar in their ability to infect mice by all routes, while the 6 BC strain failed to cause any sickness or death in this test when inoculated by the intramuscular and subcutaneous routes. Many of the mice inoculated by these routes with the egret and Borg strains exhibited signs of weakness and roughness but did not die in the 20-day period of observation. The sporadic nature of the deaths following intramuscular and subcutaneous inoculation make them unsuitable for calculating the LD_{50}, but this was done anyhow to present a rough index of infectivity. Elementary bodies could be demonstrated in the livers and spleens of mice dying after peripheral inoculation.

TABLE 2.—*Virulence for mice by various routes of inoculation.*

Virus	Route of inoculation			
	Intracerebral	Intraperitoneal	Intramuscular	Subcutaneous
6 BC	6.5*	5.0	No deaths	No deaths
Borg	5.8	5.3	4.0	2.0
Egret 1950 (A)	6.4	5.8	2.8	2.7
Egret 1950 (B)	7.6	7.5	4.0	2.7
Egret 1951	7.2	5.5	3.6	1.5

* Expressed as the logarithm of the reciprocal of that dilution of tissue calculated to be one LD_{50} according to the method of Reed and Muench.

Guinea pigs.—A sharp rise in temperature was invariably noted in guinea pigs inoculated with the egret or Borg strains by the intraperitoneal route, and death usually followed in 6 to 8 days. Intracerebral inoculation of these strains was rapidly fatal. The standard 6 BC strain, although causing a brief rise in temperature after intraperitoneal inoculation, only rarely caused the death of a guinea pig. Meyer and Eddie have corroborated this finding.[3]

The similarity of the pathogenicity of the egret and Borg strains for mice and guinea pigs is the strongest evidence obtainable at present for the hypothesis that these are the same agents or very closely related ones.

Toxins

Association of a toxin with various members of the psittacosis-lymphogranuloma venereum group was first reported by Rake and Jones,[5] and these findings were expanded by Manire and Meyer.[6] It was considered of interest to establish the presence of such a component with the egret virus, and to investigate its relationship to other viruses in the group which had already been studied.

4. Reed, J. and Muench, H. 1938, Am. J. Hyg. **27**: 493–497.

5. Rake, G. and Jones, A. P. 1944, J. Exper. Med. **79**: 483–486.

6. Manire, H. P. and Meyer, K. F. 1950, J. Infect. Dis. **86**: 226–232.

To titer the toxic activity of the egret strains, twofold dilutions from 1:10 through 1:160 were made in a 0.1 *M* phosphate buffer containing 0.2% gelatin, and 0.5 cc was inoculated into the tail veins of groups of five and six mice. Mice dying 40 hours or earlier following intravenous inoculation were scored as toxic deaths, and those dying later were arbitrarily classified as deaths due to infection.

Most of the animals inoculated with dilutions ranging from 1:10 to 1:40 died within 40 hours when either the 1950 or 1951 egret strains were used. The 1:80 dilution sometimes caused no deaths within 40 hours, but more often resulted in quick death in from 1 to 5 mice in the inoculated group of 5. This variation was due in part to the variation in size of the mice, and also in freshness of the material used, the toxin being highly labile.

Those mice which survived the 40-hour period began to exhibit symptoms of infection early thereafter and all died within 5 days. Thus, a two-phase death curve for toxic and infectious deaths, respectively, was demonstrated, which was similar to that noted by Manire and Meyer[6] for the Louisiana pneumonitis (Borg) and Illinois meningopneumonitis viruses. Table 3 illustrates the two-phase nature of the deaths, and suggests a relationship to other strains known to exhibit such a pattern.[6] It might be noted, however, that the peak observed for both toxic and infectious deaths occurred 10 to 20 hours later than that previously reported for Louisiana pneumonitis (Borg) virus. It is obvious, though, that the interval between intravenous inoculation and death is a function of the concentration of unaltered toxin, which is highly labile, and of the size of the animals inoculated. Therefore, differences of a few hours in incubation period reported on different preparations of a single virus in different laboratories are to be expected.

Toxin and intranasal infectivity neutralization tests

In further attempts to unequivocally establish the relationship of the egret strains to the Borg strain and to other members of the psittacosis-lymphogranuloma group, the toxin neutralization test, as modified by Manire and Meyer,[7] and the intranasal neutralization test of Hilleman[8] were applied with the four strains available to us.

7. Manire, G. P. and Meyer, K. F. 1950, J. Infect. Dis. **86**: 241–250.
8. Hilleman, M. R. 1945, J. Infect. Dis. **76**: 96–114.

TABLE 3.—*Toxins associated with the egret viruses deaths in mice following intravenous inoculation.*

	Total inoculation	Toxic death 40 hours*	Death associated with viral multiplication				Survived to 120 hours
			60 hours	80 hours	100 hours	120 hours	
1950 egret strain							
1:10	8	6		1	1		
1:20	11	9		1	1		
1:40	12	8		1	3		
1:80	16	8		2	3	3	
1:160	11	3		1	2	3	2
1951 egret strain							
1:10	12	10	1	1			
1:20	10	6		1		3	
1:40	12	5	1	1		4	1
1:80	11	1		2	2	4	2
1:160	12			1	3	3	5

* Death occurring before 40 hours is here considered a toxic death. Later deaths attributed to the effects of viral multiplication.

The results of one toxin neutralization are shown in table 4.

It was not possible to differentiate between available strains using either test, although it was evident that neutralization of the toxins and of the infectivity of the viruses by the intranasal route could be obtained with antiserums to any one of the four strains, as shown in table 4.

TABLE 4.—*Effect of antiserums on mortality due to egret virus toxin.*

Challenge toxin	Antiserums				
	Anti-1950 egret	Anti-1951 egret	Anti-Borg	Anti 6 BC	Control (saline)
1950 Egret	*0/6	2/6	0/6	1/6	5/6

* Numerator = number of toxic deaths, denominator = number of mice inoculated.

Serum survey of Louisiana birds

Available in the laboratory were a number of serums which had been collected from wild birds in the Louisiana swamps at the same time that the 1950 egret strain was obtained. To obtain a quantity sufficient for complement fixation, the serums had to be diluted four- and eightfold. Of the 24 American egrets, 20 snowy egrets, 8 brown pelicans, 5 Louisiana herons, 3 little blue herons, 2 anhinga, and 2 boat-tailed grackles tested by direct and indirect techniques, not one proved positive. One can conclude either that the virus was not widely prevalent in these species in 1950, or, more likely, that the antibody response was of such a low level as to be undetectable when these serums were diluted four- to eightfold, since low antibody titers are common in birds infected with viruses of this group.[9,10]

Antibiotic sensitivity

Most of the psittacosis strains of human and psittacine bird origin have been found to be unaffected by the action of sulfadiazine, whereas many of the strains from other sources are susceptible to its action and are considered to be part of the ornithosis or pigeon group.[3] It was therefore decided to compare the effectiveness of this drug on infection with the Borg strain and on the newly isolated agents in chick embryos. Also included was a comparative titration of the inhibitory effect of penicillin, aureomycin, and terramycin on these agents, with the double intent of finding an effective therapeutic agent and of studying any differences or similarities which might throw some light on the relationship of the Borg and egret strains. Yolk sac suspensions of each virus were diluted to 10^{-5} in saline, the dilution estimated to contain 100 to 1000 egg LD_{50}. The antibiotic, in amounts designated in table 5, and the diluted virus were inoculated simultaneously into the yolk sacs of 7-day-old chick embryos. These were examined daily, and the day of death recorded. Those dying before the third day were listed as nonspecific deaths. In some cases, a number of the embryos which received aureomycin or terramycin survived, and no average day of death could be determined for the entire group of embryos; in these cases, the average day of death is noted for those which died, and reference is made in the footnote to the number of survivors.

Sulfadiazine exerted little, if any, effect on the course of infection with any of the agents studied, indicating that they were truly "classical" strains of psittacosis.[3] Penicillin prolonged the incubation period from 2.3 to 4.0 days, depending on the dosage used. Aureomycin and terramycin were the most effective antibiotics against the egret

9. Davis, D. 1947, Pub. Health Rep. **62**: 1484–1488.
10. Davis, D. 1950, J. Am. Vet. M. A. **116**: 220–223.

TABLE 5.—*Inhibitory effect of antibiotics in viral infections in chick embryos.*

Virus	Average day of death*							
	Control (saline)	Sulfadiazine 1.0 mg	Penicillin		Aureomycin		Terramycin	
			250 units	1000 units	0.25 mg	1.0 mg	0.25 mg	1.0 mg
Borg	3.7	4.6	6.5	7.6	4.6	7.7	5.3	7.8
Egret 1950	4.6	5.0	7.2	7.7	9.0	10.7†	11.5‡	11.0‖
Egret 1951	5.0	5.0	7.3	9.0	9.8	12.0§	No deaths	No deaths

* A simple average of the number of days elapsing between inoculation and death, disregarding survivors from the calculation.
† 6 of 10 survived.
‡ 6 of 12 survived.
‖ 9 of 12 survived.
§ 6 of 11 survived.

viruses, the latter being particularly effective in completely sparing a high percentage of the embryos. Superiority of either to penicillin was not evident in tests with the Borg virus. In this connection it might be noted that the concentration of the Borg virus used was probably greater than that of the other viruses, as evidenced by a shorter incubation period among the controls. This difference noted between the Borg and egret strains may be attributable to the apparently greater concentration of the Borg virus used or to a deficiency in the reliability of the test, since only one trial was conducted; or it may actually be a reflection of a real difference between the strains.

DISCUSSION

The possibility that the epizootic reported here originated from a laboratory infection cannot be entirely eliminated. However, every precaution was taken to minimize the danger of such an occurrence. This fact, taken together with the observation that the 1950 strain was obtained from apparently healthy young egrets in their native habitat, lends weight to the thesis that one or more of the birds was already harboring a subclinical infection upon arrival at the laboratory. The radical change in living conditions from those encountered in their natural surroundings to those inherent in cage life at the laboratory could easily have disturbed the host-virus relationship in the birds and could have triggered the latent virus to an overt infection. According to Meyer, "latent, inapparent infections represent the corner-stone of the entire psittacosis and ornithosis problem."[11]

The recovery of a virus of the psittacosis-lymphogranuloma venereum group from wild egrets from southeastern Louisiana for two successive years may be a fact of considerable public health significance. This agent, in its virulence for certain hosts by various routes, high toxicity, spectrum of sensitivity to antibiotic, and the general geographic locale of origin, seems similar to, if not identical with, the agent which was responsible for a severe epidemic of human pneumonitis reported in the same general area in 1943.[3] While it is true that in the aforementioned epidemic all cases subsequent to the first one could be ascribed to contact with an infected person, the source of infection in the first case remained unknown. This original case occurred in the wife of a trapper, living in a bayou region with an abundant bird population. Many species of birds, including the legally protected egrets, are shot and eaten by the local population, and such intimate contact could easily have led to the transmission of the organism.

There have been no recorded instances since 1943 of severe human in-

11. Meyer, K. F. 1942, Medicine, **21**: 175–206.

fections in this area, similar to those occurring during the epidemic. How can this be explained, in view of the fact that a virus very similar to, if not identical with, that responsible for the epidemic has been demonstrated to be currently present in the bird population of that vicinity? One possibility is that the widespread practice of giving "prophylactic" inoculations of penicillin and the newer antibiotics early in the course of febrile respiratory disease may have masked some of the cases. Another is that a mutant or recombinant of this virus with particular virulence for man was responsible for the 1943 series of cases, and the egret virus, in its present form, is not capable of causing a similar reaction unless a similar variation occurs. Considering the extreme toxicity of the egret virus for experimental animals reported here, however, one would expect at least some degree of pathogenicity for humans.[12] The possibility that this virus is currently responsible for milder atypical pneumonias of obscure origin in this region cannot and should not be ignored.

The current role of the egret virus, if it has such a role, in causing respiratory disease of unknown etiology among people in contact with wild wading birds can be determined only by serum surveys of the local populations in these areas, and by increased efforts to determine the causative agent or agents in the obscure respiratory disease occurring there.

12. Meyer, K. F. Personal communication.

SUMMARY

A highly fatal epizootic is reported in wild American and snowy egrets captured in southern Louisiana and kept in the laboratory. A virus of the psittacosis-lymphogranuloma venereum group was isolated from eight of the birds, and it proved to be identical in all its characteristics to an agent isolated a year before from two snowy egrets bled in their native habitat. This agent also proved to be indistinguishable from that of Louisiana pneumonitis (Borg), which originated in a severe human epidemic in southern Louisiana in 1943. The possibility is pointed out that a potential public health menace exists in areas which serve as the natural habitats of egrets and, perhaps, of other species of wading birds.

STUDIES ON THE EXPERIMENTAL EPIDEMIOLOGY OF RESPIRATORY INFECTIONS

VI. THE RELATIONSHIP BETWEEN DOSE OF MICROORGANISMS AND SUBSEQUENT INFECTION OR DEATH OF A HOST

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Primary pneumonic infection may be produced in animals exposed to an aerosol of a suitable pathogen. It is logical to assume that the numbers of organisms inhaled per unit of time, and the duration of such an exposure, would influence the number of animals so affected. This in turn may be expressed in a mathematical relationship using the variables, dosage and mortality.

It is our object to present such a mathematical formulation, based on a physical model, relating dosage to mortality. In order to ascertain the validity of this formulation, a series of experiments was designed to obtain the relationship between magnitude of dose of a respiratory pathogen and subsequent mortality.

When an arbitrary exposure period of 20 minutes was selected for challenging test groups of Namru strain of albino mice (Garber and Hauth, 1950) with various respiratory dosages obtained by altering the concentration of the organisms in the suspension to be atomized, the observed mortalities roughly paralleled the aerosol concentrations. This indicates that a definable relationship may be demonstrated between dosage and mortality. The response of the host when the same number of organisms is given in a different time pattern was also explored to determine the applicability of a given survival relationship. *Klebsiella pneumoniae*, *Streptococcus zoepidemicus*, *Pasteurella pestis*, and the Cal-ten strain of meningopneumonitis virus were studied, using albino mice as the test host. Application of the mathematical relationship to other organisms appearing to date in the published literature was made in an attempt to establish universality.

MATHEMATICAL CONSIDERATIONS

To obtain the critical data upon which a generalized formulation could be constructed, certain basic assumptions relating dosage, mortality, and exposure were made.

First, it was assumed that a single inhaled bacterial cell, if conditions were favorable, would result in an infection or death of the host. Second, it was assumed that death of an animal following inhalation of a single cell would occur with a measurable probability, i.e., the probability of this one cell reaching an environment favorable for its multiplication to the point where disease and subsequent death would occur. Third, it was assumed that organisms were inhaled in discrete particles slightly larger than the bacterial cell, no particle containing more than one infective unit. Fourth, it was assumed that each cell, when more than one was inhaled, would

Received for publication May 6, 1953.

This work was supported by a contract between the University of California Department of Bacteriology and the Office of Naval Research.

The opinions contained in this report are not to be construed as reflecting the views of the Navy Department or the Naval Service at large (Article 1252, U. S. Navy Regulations, 1948).

behave independently of other cells in its pathogenic potential. To illustrate, if a mouse's chance of survival following inhalation of a single pathogenic cell were 0.99985, then its chance of surviving a multiple inhaled dosage of n organisms would be $(0.99985)^n$.

This relationship can be restated in terms of symbols: Let S_d be the probability of survival following a dose of d organisms. Let S_1 be the probability of survival following a dose of 1 organism. In symbols, this relationship can be expressed:

$$S_d = (S_1)^d \qquad 1(a)$$

Now let d assume an experimental value, d_0, and let S_{d_0} also be observed experimentally. We have:

$$S_{d_0} = (S_1)^{d_0} \qquad 1(b)$$

We have, however,

$$(S_d)^{1/d} = S_1 = (S_{d_0})^{1/d_0},$$
$$(S_d)^{1/d} = (S_{d_0})^{1/d_0},$$

or

$$S_d = (S_{d_0})^{d/d_0} \qquad 1(c)$$

This form allows one to compute directly any S_d from an observed S_{d_0} which resulted from the dose d_0.

If one wishes to re-express equation 1(c) in terms of mortality rather than survival, we have:

$$(1-M_d) = (1-M_{d_0})^{d/d_0} \quad \text{(equation 1)}$$

It is to be noted that neither the number of viable cells inhaled per unit time nor the time pattern nor period of exposure are involved directly in the above assumptions. This particular point was tested experimentally.

However, if the possibility of immunity is considered, then the period of exposure must be included for consideration. This follows from the observation that an immune response occurs in the host after a period of time following a given exposure. Hence, there is a definite chance of an animal receiving an early immunizing nonlethal infection which would afford protection against future exposure, thus altering the effectiveness of multiple dosage, or otherwise varying the response. Such a situation actually occurred with *K. pneumoniae* after 6 to 8 weeks of daily low-grade exposures, but no alteration in response was noted during the first month.

Since it is advantageous to be able to present the above relationship (equation 1) graphically as a straight line, a special coordinate paper called the "Linearized Mortality Grid" (LMG) has been developed (fig. 1).

Starting with equation 1,

$$(1-M_d) = (1-M_{d_0})^{d/d_0},$$

and taking the log log of both sides, we obtain:

$$\begin{aligned}\log\log(1-M_d) &= \log\log\left[(1-M_{d_0})^{d/d_0}\right],\\ &= \log\left[d/d_0 \log(1-M_{d_0})\right],\\ &= \log d + \left[\log\log(1-M_{d_0}) - \log d_0\right].\end{aligned}$$

Now let

$$X = \log\log(1-M_d)$$
$$Y = \log d$$
$$C = \log\log(1-M_{d_0}) - \log d_0$$

We have: $X = Y + C$, which is the equation of a straight line with unit slope.

If the abscissa is measured in units of log log $(1\text{-}M_d)$ and the ordinate in units of log d, a graph of equation 1 on such a coordinate grid will appear as a straight line. For convenience, the coordinates are labelled in terms of mortality and dosage, since these are the quantities of direct observation.

The scale of log d was chosen in preference to a linear dosage scale so that one could conveniently represent a wide

range of dosage values with equal accuracy on all portions of the scale.

If one arbitrarily selects values for M_{d_0} for a given d_0 and plots the relationship given by equation 1 on the LMG, a member of the infinite family of curves which satisfies equation 1 is generated. Reference diagonal lines generated in such a fashion are given on the LMG to act as plotting guides.

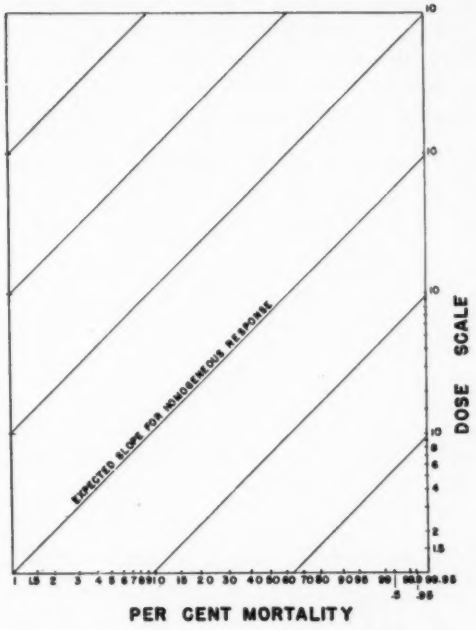

FIG. 1.—Linearized mortality grid used for relating infective or lethal responses with challenging dose, showing expected slope for homogeneous response.

To test these implications experimentally, two modes of host exposure to aerosols were used: (1) a total single exposure period of less than 1 hour, producing mortalities over a range of 0 to 100%; changes in total dosage were affected by changes in rate of exposure during the test period; (2) a 40-minute exposure, resulting in a nominal inhaled dosage of 70 organisms per mouse, repeated daily for as long as 7 weeks; in this case, the daily average mortality should be consistent with the mortality expected following inhalation of 70 organisms.

METHODS

A. K. pneumoniae

A suspension of organisms of *K. pneumoniae* for atomization and serial passage was prepared daily as follows: Nine mice were each inoculated intranasally with 0.05 ml of a suspension of the organism grown in tryptose-phosphate-infusion broth (TPI). The inoculum contained approximately 1×10^7 organisms. Within 24 hours most of the mice were dead. Lungs and hearts from 6 dead mice were removed aseptically and ground in a mortar with the addition of 9 ml of sterile Alundum grinding compound. Eighteen ml of TPI were then added to the mortar and mixed thoroughly. After settling of the larger particles, the supernate was pipetted to a sterile test tube. At this point, a 5% horse-blood agar plate was streaked and incubated 24 hours as a purity check. The test tube was then placed in a refrigerator, precautions being taken to keep it vertical so that settling material reached the bottom and did not adhere to the walls. Approximately 16 hours later 9 ml of the supernate were placed in a small flask and agitated 10 to 20 times by a 10 ml pipette to ensure disaggregation of the organisms. The suspension was then diluted with fresh TPI to the bacterial concentration desired for a given exposure. In the case of the daily exposures, the suspension was diluted to 1/100 with TPI. To expose the animals, 100 ml of this cell suspension (1 to 4×10^7 viable cells/ml) were atomized for a period of 40 minutes.

Exposure groups of approximately 200 mice each (initially 8 to 12 weeks old) were held in units of 15 to 16 mice per cage. The groups were exposed serially as follows: Occupants of a given cage were always distributed among the same 4 subdivision cells of a channel (fig. 2) in order to evaluate the possible effect of exposure position. Since the drop in bacterial viability was observed to be less than 10% at the distal end of the channel, it was not necessary to correct the observed mortality for this factor.

The aerosol was generated by using a Wells-type atomizer operated at 8½ p.s.i (Leif and Krueger, 1950). A total of 6 cubic feet per minute of filtered room air was mixed with the atomizer output and then divided into flows of 1 cubic foot per minute per exposure channel. A 15-minute minimum air-wash time was interposed between the exposure period and unloading, 5 minutes having proved adequate to reduce the cage aerosol concentration to less than 1 organism per cubic foot of contained air.

The cloud concentration was determined by means of an air slit sampler (Goldberg and Schechmeister, 1951) operated at a collection rate of 4.2 liters per minute, using both nutrient and blood agar plates. A "photoelectric aerosol-concentration monitor"* was introduced into the system to indicate possible changes in atomizer output during an exposure period.

Mice dying during the experiment were autopsied; a streak from lung tissue was made on 5% horse-blood agar plates. The typical pearly-appearing mucoid colonies which trailed strands of material and stained as gram-negative capsulated rods were considered to be *K. pneumoniae*. The dead mice were removed from the cages during several inspections, made over a 24-hour period, in order to minimize cannibalism. There were no significant differences, however, in mortality between cages where cannibalism had or had not occurred.

B. Str. zooepidemicus

The preparation of *Str. zooepidemicus* suspensions proceeded along somewhat different lines: The organism was not maintained by animal passage but was taken as needed from 3% cow-blood agar slants. Three such slants were each washed with 2.5 ml TPI, the washings then being inoculated into flasks containing 50 ml TPI and 2.5 ml cow serum. These flasks were then incubated at 37 C for 8 hours. A second passage was then made by transfering 5 ml to each of another set of flasks of the same medium. The first flasks were checked for purity of culture. The second set of flasks was incubated at 37 C for 16 hours. At this time the viable assay was 5×10^8 to 1×10^9 organisms per ml. The three flasks were then pooled, refrigerated and used within one week. For use, the culture was diluted with fresh TPI containing 5% cow serum to a bacterial concentration desired for a given exposure. In the case of the daily exposures, 6 ml of this pooled culture were diluted to 150 ml with refrigerated TPI and 5 ml cow serum and this further diluted tenfold in Sorenson's phosphate buffer solution, pH 7.0, when used for atomization.

In an experiment using *Str. zooepidemicus*, 509 mice were employed. The mice were subdivided by age into classes of 5 to 6 weeks, 6 to 7 weeks, 7 to 8 weeks, 8 to 9 weeks, and approximately 32 weeks. Four replicate groups were formed, using 32 mice each from the first three age classes and 16 mice each from the latter two. These four groups were exposed daily, 6 times per week, for a period of 5 weeks. Three percent cow-blood agar plates were used as the collection medium for cloud sampling. All mice dying during and for 2 weeks subsequent to the exposure period were autopsied, and lung cultures were made to demonstrate the presence of the organism. No nonspecific deaths occurred.

In parallel experiments using *Str. zooepidemicus*, 6 groups of 32 mice each were given single doses of the organism in either a 6-hour or 15-minute period. Fivefold dilutions of the suspension were made to result in a nominal 15,000, 3000, and 600 organisms inhaled per group for each of the dose-exposure times. The air was sampled either by means of the slit sampler or by a capillary impinger (Leif and Krueger, 1950) in order to evaluate the inhaled dose per mouse during the exposure.

C. Past. pestis

The preparation of *Past. pestis* followed a procedure similar to that used for *Str. zooepidemicus*, with the following points of difference: The culture medium was HIB (heart infusion broth, Difco), each of the two passages was for 24 hours, and the stock slants were made using horse-blood agar. Routine plate counts were done on 0.5% horse-blood agar plates. The exposure technique and equipment used have been described by Leif and Krueger (1950).

D. Cal-ten strain of meningopneumonitis virus

Meningopneumonitis virus, originally obtained from the Virus Research Laboratories, California State Department of Health, was maintained by passage in the allantoic cavity of embryonated eggs and in lungs of mice infected intranasally (Elberg et al, 1948). Infected allantoic fluid, and later nutrient broth suspensions of infected mouse lungs, were used as spray suspensions to produce viral aerosols. Exposure time was 20 minutes by the techniques described above.

Assay of clouds, taken in capillary impingers charged with nutrient broth, was carried out by the intracerebral inoculation of mice, 6 mice each receiving 0.03 ml of the appropriate dilution of impinger fluid.

Concentration of virus in this impinger fluid was then expressed as that volume of the original fluid which gave a computed 50% kill in the animals used for titration.

RESULTS

The results of a series of experiments of the 4 pathogens cited, involving single exposure times of 1 hour or less,

* A modified Tyndall meter, using a 931-A photocell.

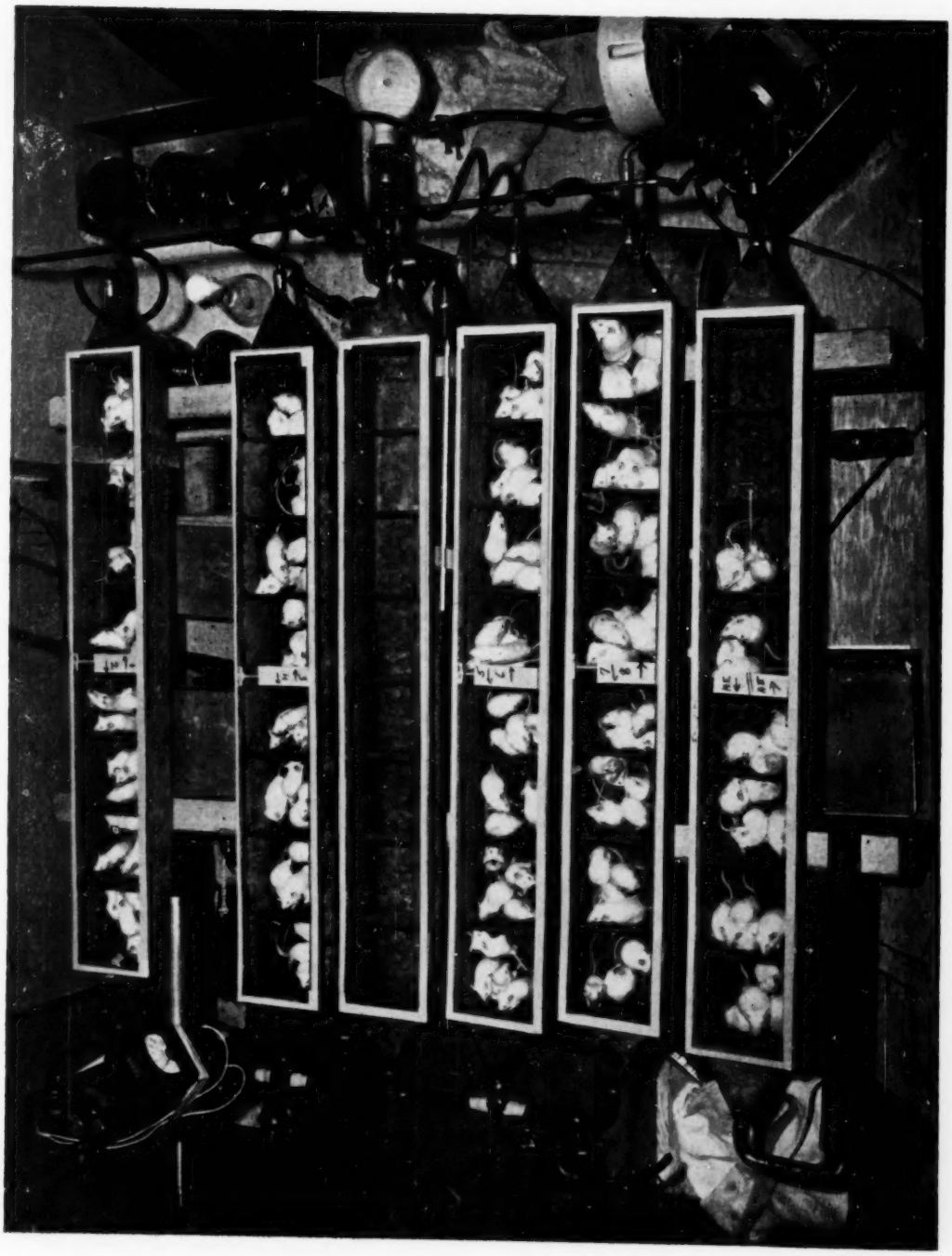

FIG. 2.—Exposure apparatus.

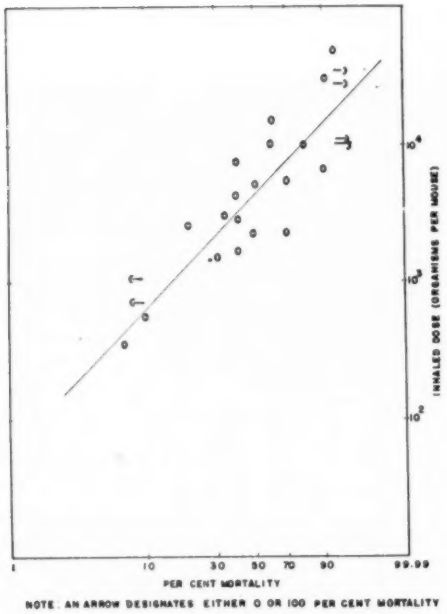

FIG. 3.—The dosage-mortality relationship for albino mice (Namru strain) infected with *Past. pestis* by the respiratory route, when exposure time was less than 1 hour, in groups of 10 to 16 animals.

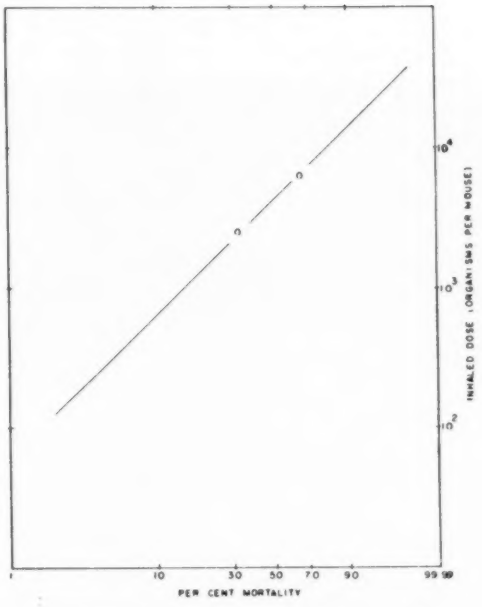

FIG. 4.—The dosage-mortality relationship for albino mice (Namru strain) infected with *K. pneumoniae* by the respiratory route, where exposure time was less than 1 hour, in groups of 288 animals.

are given in figures 3, 4, 5, and 6. In addition, figure 5 gives data for a 6-hour exposure. It is seen that the plot of the data on the LMG results in an acceptable fit.

It should be noted that an arrow on the graph indicates that either 0 or 100% response resulted from a given test dose. The arrow is placed at a point on the graph corresponding to the initial group size and pointed in the direction of the indicated response. For example, when 0 or 100% response is observed, where 10 animals were exposed, arrows are placed at the 10 and 90% ordinates (fig. 3); however, when groups of 32 animals are exposed, arrows are placed at the 3 and 97% ordinates, respectively (fig. 5).

The results from experiments with *Str. zooepidemicus* and *K. pneumoniae* involving 40-minute daily exposures, using an average daily inhaled dose of 60 to 70 viable organisms per 20-g

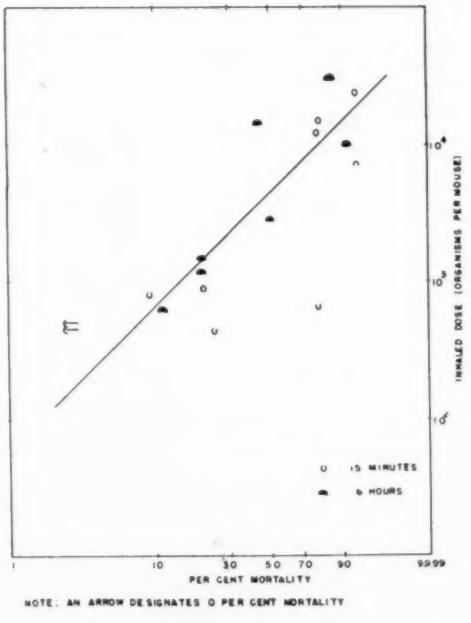

FIG. 5.—The dosage-mortality relationship for albino mice (Namru strain) infected with *Str. zooepidemicus* by the respiratory route, where exposure times were 15 minutes and 6 hours, in groups of 32 animals.

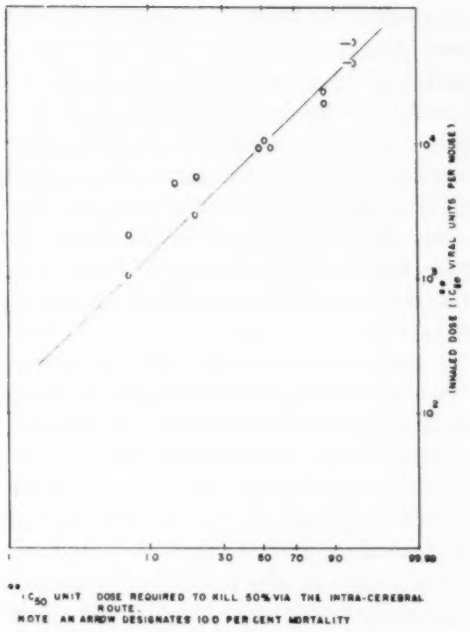

FIG. 6.—The dosage-mortality relationship for albino mice (Namru strain) infected with the meningopneumonitis virus, Cal-ten strain, by the respiratory route, where exposure time was less than 1 hour, in groups of 10 to 16 animals.

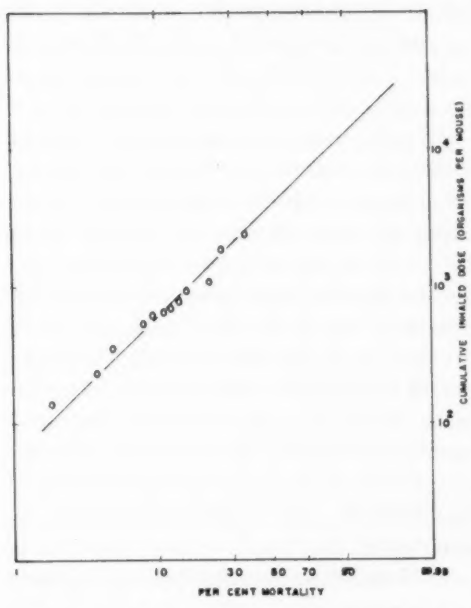

FIG. 7.—The cumulative dosage-mortality relationship for albino mice (Namru strain) infected with *Str. zooepidemicus* by the respiratory route, using a 40-minute exposure, repeated daily for a period of 1 month, in a group of 192 animals.

mouse, are given in figures 7 and 8. Comparison of figure 5, which describes data obtained from a single heavy challenge, with figure 7, giving deaths from the same cumulative dose over a 30-day exposure, shows that the total mortality in either case is identical.

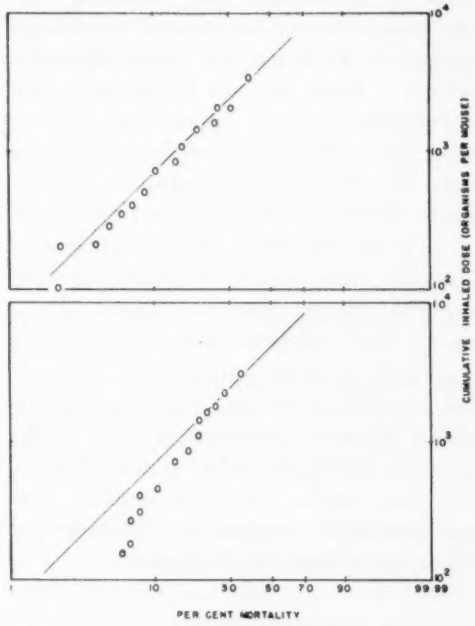

FIG. 8.—The cumulative dosage-mortality relatilnship for albino mice (Namru strain) infected with *K. pneumoniae* (type A) by the respiratory route, using a 40-minute exposure, repeated daily for a period of 1 month, in a group of 240 animals.

A comparison of the results obtained with *K. pneumoniae*, *Str. zooepidemicus* and *Past. pestis* indicates complete agreement on the LRE_{50}* values of 4 to 5×10^3 organisms inhaled, regardless of bacterial species. This is equivalent to an expected mortality of 0.00015 per inhaled organism. It is to be noted, however, that the value obtained from the virus data is 1×10^4 as measured in IC_{50} units and is not directly comparable to bacterial assay.

Data on age-mortality results, using

* The numbers of inhaled organisms causing the death of 50% of the exposed animals.

Str. zooepidemicus, showed no significant differences in observed mortality when the mice of various age groups were exposed daily to the same aerosol.

These quantitative data demonstrate that a definable relationship exists between dosage, mortality and exposure time. In particular, they indicate that the mortality can be related to dosage (equation 1) when the total exposure period is short enough to preclude the development of an appreciable immunity. Mortality, as given by this equation, is expressed in terms of dosage alone, and hence is not a function of rate or time of exposure. Results observed, however, from exposures extending over a period longer than one month, have shown that the time period and rate of exposure become important. This is presumed to coincide with appearance of an immune response several weeks after an exposure takes place. Studies are now under way to modify this mathematical expression to include the effect of an immune response.

DISCUSSION

It is possible to draw the inference from the dosage-mortality relationship we have described that a single inhaled organism will result in a small, but measurable, probability of death. Taking equation 1 and an LRE_{50} value of 4600 organisms and solving for M_{d_0} when $d_0 = 1$, we have:

$$1 - M_d = 0.5 = (1 - M_1)^{(4.6 \times 10^3)},$$

$$1 - M_1 = 0.99985,$$

$$M_1 = 0.00015. \qquad \text{(equation 2)}$$

To observe such an effect directly one would have to expose a minimum of 6700 mice, so that each one received a minimal or unit-inhaled dose. Although it has not been found possible to carry the results out to this limit, data have been presented for an inhaled dose of 70 organisms per mouse, and the results were found consistent with the value expected from equation 1. It should be noted that the retained lung dose was only 7 organisms (Goldberg, 1950) and consequently approached the theoretical limit of unity. This result indicates that a so-called "minimum lethal dose," or dose below which no mortality is evidenced, is, in all probability, one bacterial cell.

If an animal inhales 1000 organisms, the *mortality expectancy computed*, using equation 1, is 14% where M_{d_1} is assumed to be 0.00015. If one multiplies the expected mortality per inhaled organism by 1000, one obtains 15%, as compared to the computed 14%. Allowing this approximation, one is led to an interesting concept which may be applied when a detailed knowledge of the distribution of inhaled dosage is lacking, as would probably be the case in studies in the air-borne infections in experimental herds: Let the reciprocal of 0.00015, which is the probable mortality expected per single inhaled cell, be designated as a lethal unit, L_x, where x can be used as the pathogen designation.

In this example, if no animal receives a dosage greater than 1000 organisms, or a dosage which would result in an expected mortality of no greater than 14%, then the number of deaths expected in a large group of animals which inhales a total of N organisms is N divided by L_x. In other words, the number of lethal units inhaled by the group as a whole is equal to the expected number of deaths. This data can be used to determine the constants involved in equation 1, since one need only to determine:

1. Total dose inhaled by the exposed group.
2. Percent mortality in the exposed group.
3. That the maximum inhaled

dose/animal at risk is within allowed limits, i. e., mortality expectancy of less than 15% would result from any inhaled dosage.

The entire dose-mortality relationship may be determined from the above data. Conversely, if the dose-mortality relationship is known for a given pathogen, and if a group of animals is exposed, the percent mortality which occurs allows an estimate to be made of the number of L_x units that are present in the total inspired air.

Desiring to determine how the data of other investigators would fit the LMG or equation 1, we turned to the herd studies of Webster at the Rockefeller Institute (Webster, 1928). In this work he used Rockefeller Institute (RI) and the Lathrop strain of mice for epidemiological studies involving herds of mice, with several members of the herd initially infected with *K. pneumoniae*. (It is to be noted that this strain of the organism could not be typed with the usual typing serums.) Additions to the herd under study were made daily, using previously uninfected, or nominally clean, mice. In the series of experiments in which RI mice were used exclusively, the epidemic outbreaks would occur for a period but would eventually damp out. If the Lathrop strain of mice was introduced, the damping would no longer be observed and outbreaks of mortality reoccurred after a sufficient accumulation of susceptibles.

Upon examination of a series of intranasal titrations which he reported using RI mice and his strain of *K. pneumoniae* (fig. 9), one sees that 10% of the exposed group survived regardless of the intranasal dosage involved. This appears graphically as the vertical line at 90% response. If this resistant fraction or subgroup were allowed to build up in a herd by the selective mortality which would occur, after a period of time it would constitute such a large fraction of the test herd as to damp out any further outbreaks among the susceptibles. This effect could be eliminated or reduced by limiting cage life to a given arbitrary time, after which a given animal would be removed and replaced.

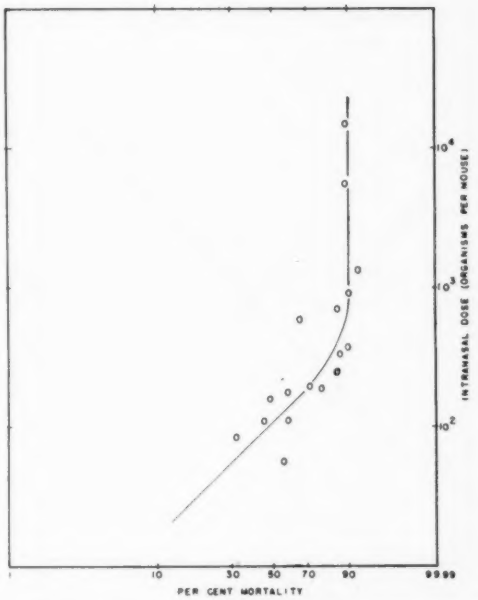

FIG. 9.—The dosage-mortality relationship for the total tested population of Rockefeller mice, infected with *K. pneumoniae* (strain B) by the intranasal route (from data of Webster, 1928).

To further test the applicability of the equation

$$(1-M_d)=(1-M_{d_0})^{d/d_0}$$

we turned to the dose-mortality response of viruses in the chick embryo, as reported by Crawley (1948), using a strain of Eastern equine encephalomyelitis virus, by Bang (1948), using the Newcastle virus, and by von Magnus (1951), with influenza A virus. Crawley's data comparing two routes of inoculation and the effect of incubation temperatures were tested on the LMG. Figure 10 is a composite plot of these data to demonstrate the applicability of the LMG. A comparison of the LD_{50}

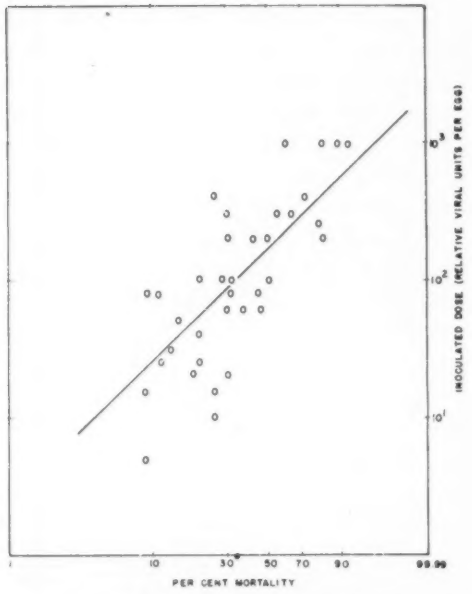

FIG. 10.—The dosage-mortality relationship for embryonated eggs infected with Eastern equine encephalomyelitis virus, using 10 embryonated eggs per test dilution (from data of Crawley, 1938).

values we obtained with those reported by Crawley show no real differences except in the rare cases in which the data itself was quite erratic. In these few cases we believe a better estimate of the LD_{50} value is possible by our technique, since the slope of the line of fit is assigned a priori. Comparison with the theoretical distribution expected from the use of 10 eggs per dilution indicates good agreement and gives further weight to the use of the LMG in this host-agent response.

Since log-probability paper was used by Crawley, it is of interest to plot equation 1 on such a grid, using a selected value of M_{d_0} (fig. 11). The change of slope from 2 to 4, with a mid-range value of 3, is to be noted over a 5 to 95% mortality range. This change in slope will account in part for the variation obtained when a straight line fit on a probit grid is attempted, as was done in the above article by this author.

The data of Bang (fig. 12) and of von Magnus (fig. 13 and 14) show that the relationship expressed by equation 1 is applicable to other viruses as well. It is interesting to note that the possibility of deriving this equation may be found in the former article.

Similarly, an example may be cited showing the application of equation 1 to a spore form in another animal host; figure 15 gives data on *Bacillus anthracis*, using guinea pigs as the test host (Young, 1946).

Another possibility is the comparison of two widely different routes of inoculation for a given microorganism. Figure 16, in comparison with data shown in figure 2, indicates that we have derived the same relationship for *Past. pestis* administered to mice either via the respiratory route or subcutaneously. It should be noted that the LD_{50} dose in these instances has changed from 4×10^3 to 2×10^0 without alteration of the basic mathematical expression.

Finally, since there is no theoretical reason to exclude the criteria of infection rather than death, the data of Elberg and Henderson (1948) were examined. They obtained data correlating respiratory exposure of guinea

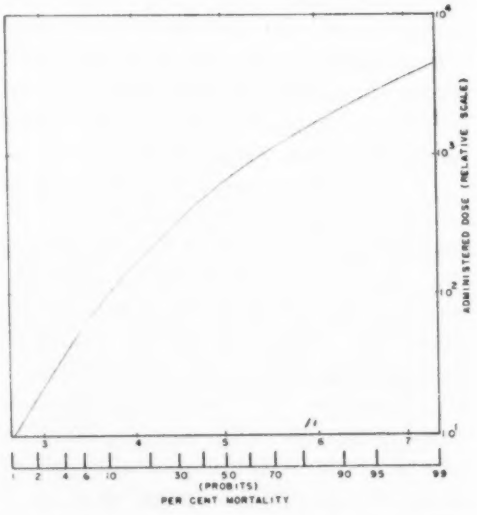

FIG. 11.—Plot of dose-mortality relationship given by equation 1 on a probit grid.

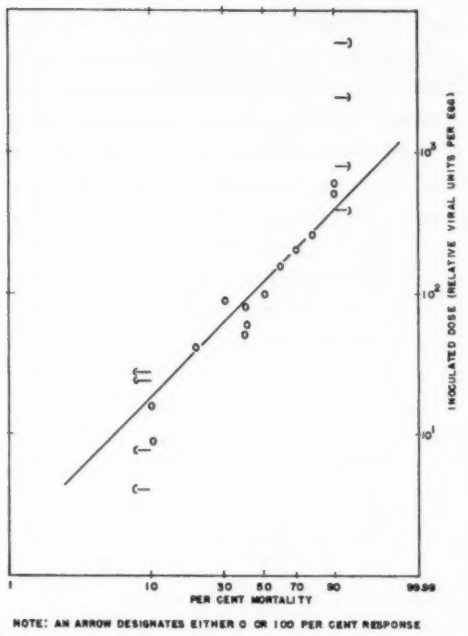

FIG. 12.—Relationship between inoculated dose and mortality for Newcastle's virus, using 10 embryonated eggs per test dilution.

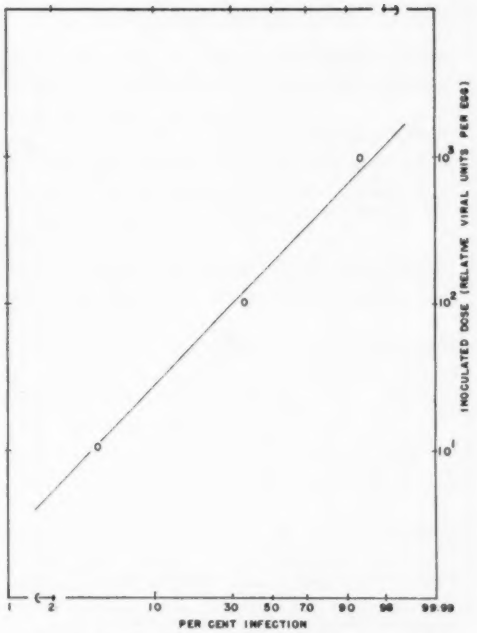

FIG. 14.—The dosage-infection relationship in embryonated eggs for influenza A (PR8) virus, using 50 embryonated eggs per test dilution (from data of von Magnus, 1951).

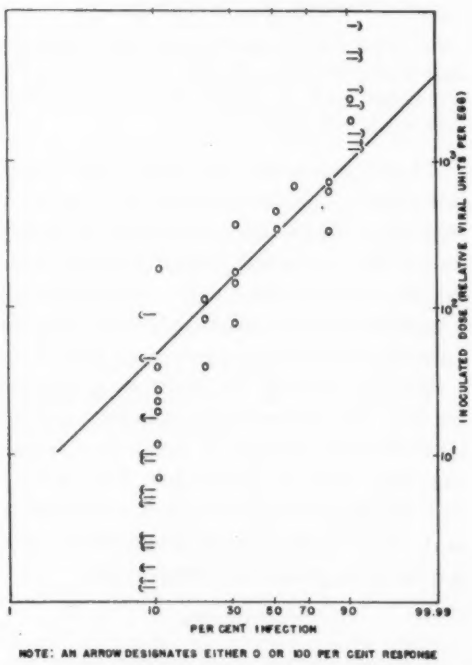

FIG. 13.—The dosage-infection relationship in embryonated eggs for influenza A (PR8) virus, using 10 embryonated eggs per test dilution (from data of von Magnus, 1951).

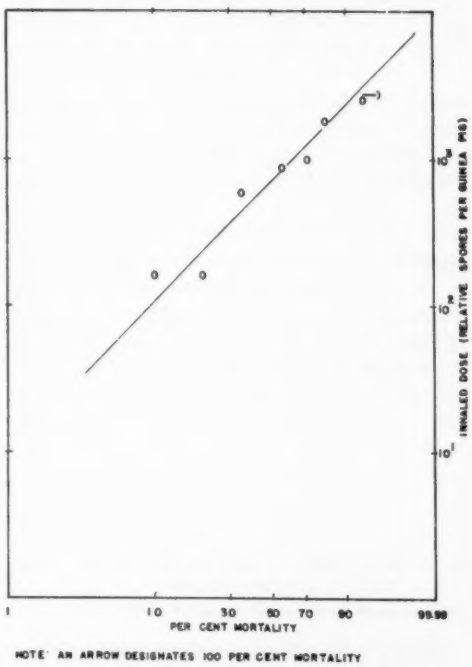

FIG. 15.—The dosage-mortality relationship for guinea pigs infected with *B. anthracis* by the respiratory route, in groups of 20 animals (from data of Young, 1946).

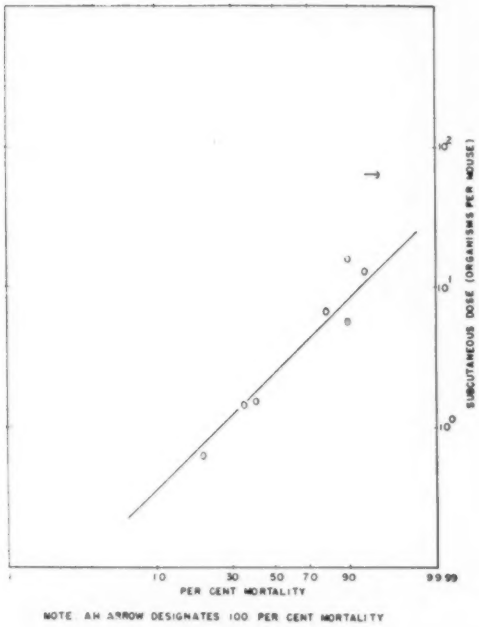

FIG. 16.—The dosage-mortality relationship for albino mice (Namru strain) infected subcutaneously with *Past. pestis*, in groups of 20 animals.

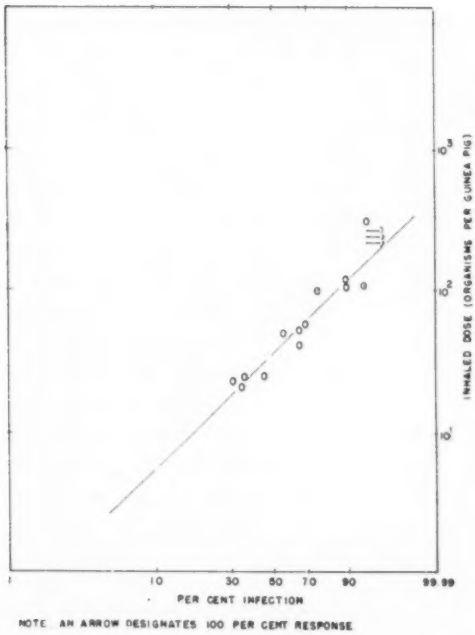

FIG. 17.—The dosage-infection relationship for guinea pigs infected with *Br. suis* by the respiratory route, in groups of 20 animals (from data of Elberg, 1948).

pigs to *Brucella suis* (fig. 17) and *Brucella melitensis* (fig. 18) with subsequent infection, as determined by autopsy performed 30 days after exposure, which also fits equation 1.

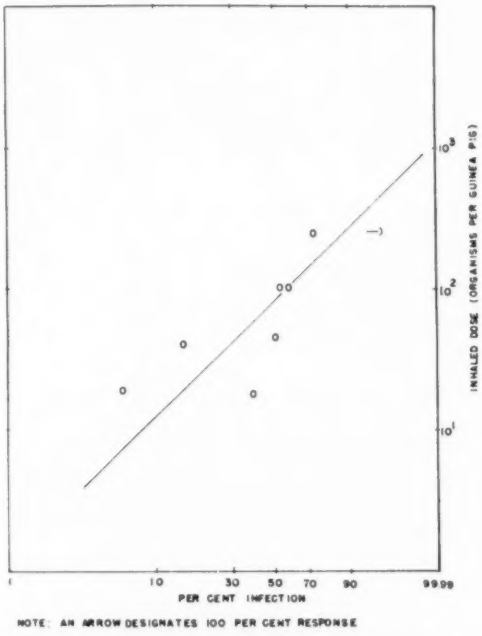

FIG. 18.—The dosage-infection relationship for guinea pigs infected with *Br. melitensis*, by the respiratory route, in groups of 20 animals (from data of Elberg, 1948).

These examples of data from our laboratory and from the literature employed 4 organisms (including a sporeform) and 4 viruses under various conditions. Three hosts were inoculated by 4 methods in cumulative lethal dosages administered in increments of less than 1 hour or as long as 30 days. In all instances, the described mathematical relationship of dosage to mortality, infection and time is applicable. We believe this strongly suggests that equation 1 may be a fundamental expression governing host-parasite interaction.

SUMMARY

A fundamental relationship is established between dosage and mortality following respiratory exposure of a

group of mice to a pathogenic aerosol. Results obtained by two modes of exposure are described: (1) one short exposure period, 15 minutes to 1 hour, using an aerosol concentration giving a range from zero to total mortality; (2) daily 40-minute exposures continued up to 4 weeks, the total dosage giving a cumulative mortality of some 40%.

The data obtained from these methods produced an empirical relationship between dose and mortality which could be expressed mathematically without the necessity of introducing any variable to account for rate of exposure. This expression is: $(1-M_d)=(1-M_{d_0})^{d/d_0}$, where M_d is the observed mortality for inhaled dose d.

The following organisms have been studied, and give data fitting this equation: *Klebsiella pneumoniae*, *Streptococcus zooepidemicus*, *Pasteurella pestis*, and meningopneumonitis virus.

Studies on cumulative-dosage and cumulative-mortality allow the above expression to apply, provided the overall time involved is not sufficiently long to permit an immune response to become a complicating factor. Periods under 30 days were satisfactory for *K. pneumoniae* infections in mice.

Additional data have been cited from the literature to illlustrate the applicability of this expression to a variety of bacterial and viral pathogens administered by several routes to several hosts. It is suggested the expression may be fundamental to the description of a parasite interaction.

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CULTURAL AND SEROLOGIC STUDIES ON NONFATAL HISTOPLASMOSIS IN MICE, HAMSTERS, AND GUINEA PIGS

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The definitive diagnosis of human histoplasmosis usually depends on the isolation of the etiologic agent *Histoplasma capsulatum*. However, in mild cases, it is often not possible to isolate the organism from or to demonstrate it in blood, gastric washings, tissue biopsies, sputum, or other available specimens. Accordingly, numerous methods have been employed in attempts to improve the chance of isolation of *H. capsulatum* from human patients. In addition, considerable research has been conducted on experimentally infected animals to determine the best methods for the demonstration of the etiologic agent.

DeMonbreun (1934) and Parsons (1942) first demonstrated that mice could be infected by injection of mycelial or yeastlike forms of the fungus. Subsequently, Tager and Liebow (1942) found that four mice 3 to 4 weeks of age, inoculated intraperitoneally with a mycelial suspension, succumbed to the infection 100 to 150 days later. Levy (1945) inoculated 145 6- to 8-week-old mice intravenously with a mixture of the yeastlike and mycelial forms of *H. capsulatum* grown for 7 days on blood agar. Subsequently, positive cultures were obtained from the livers and spleens of all the mice. The size of the inoculum and the nature of the medium used for isolation were not mentioned. Howell (1948) reported brain-heart infusion blood agar to be of value for demonstration of the fungus in the spleens of guinea pigs infected intraperitoneally "with graded doses" of the yeastlike phase. However, he did not correlate his cultural results with direct microscopic observation of the fungus in the tissues, nor did he mention the actual numbers of organisms used. Kotcher et al (1951) inoculated 42 white mice intraperitoneally with a saline suspension of ground elements of the mycelial phase, and showed (a) that *H. capsulatum* could be recovered by culture more readily than it could be identified by direct microscopic examination of stained tissue impressions, (b) that brain-heart infusion blood agar and Sabouraud's dextrose agar yielded the greatest number of positive cultures, and (c) that the highest percentage of isolations was obtained from spleens cultured on Sabouraud's dextrose agar.

None of the earlier investigators inoculated laboratory animals with accurately determined numbers of yeastlike cells. Similarly, no experiments have been recorded in which nonfatal histoplasmosis in small laboratory animals was studied with the use of serological and cultural methods. Accordingly, experiments were conducted to obtain information on (a) the response of mice, hamsters, and guinea pigs to inoculation with graded doses of yeastlike cells, (b) the possible enhancing effect of mucin in producing detectable histoplasmosis, (c) the presence of antibodies in animals inoculated with small numbers of yeastlike cells, and (d) the relative efficiencies of cultural and serological

Received for publication May 11, 1953.

The author acknowledges the technical assistance of Mr. J. C. Cory.

methods in the diagnosis of histoplasmosis.

MATERIALS AND METHODS

The mice, hamsters, and guinea pigs were inoculated intraperitoneally with yeastlike cells of *H. capsulatum* (#6515) grown for 48 hours at 37 C under constant rotation (Salvin, 1950). The medium consisted of:

Difco casamino acids (technical)	10.0 g
Dextrose	3.0 g
Yeast extract dialysate, equivalent of	3.0 g
NaCl	2.5 g
Cysteine HCl	0.5 g
KCl	2.5 g
Na_2HPO_4 (anhydrous)	4.0 g
Distilled water to make	1000 ml

The mice were all white Swiss males (Rocky Mountain Laboratory strain) 40 days old at the time of inoculation, whereas the hamsters* and guinea pigs were 30 to 40 days old and of both sexes.

The mice, hamsters and guinea pigs were each divided into two groups, one to be inoculated intraperitoneally with yeastlike cells supended in saline, the other with cells suspended in 2.5% mucin (Salvin et al, 1952). The groups of mice were divided into three lots to be given 10, 100, or 1000 cells,† and the groups of hamsters and guinea pigs each into two lots to be given 10 or 1000 cells. Except where indicated, groups of animals were bled and killed every other week after the inoculation date. The tissues (liver, spleen, and kidney) and the clots from the aforementioned bleedings were cultured in duplicate on Sabouraud's agar slants for 28 days at room temperature. Since previous workers had demonstrated the tendency of the fungus to produce macroconidia on Sabouraud's agar (Howell, 1948; Kotcher et al, 1951), and since the fungus grew as well on Sabouraud's agar as on any of the three others tested (blood agar, beef heart infusion agar, and hemoglobin cystine heart agar‡), Sabouraud's agar alone was used for culturing the animal tissues. This medium supported the growth of small numbers of cells, since the inoculation of suspensions of as low as two cells of the yeast phase resulted in the appearance of the appropriate numbers of colonies.

The serums of mice were tested only for the presence of precipitins, since the amounts of serum available were small and it was thought that precipitins might be present during the early or acute phase of infection (Salvin and Hottle, 1948). Although both complement-fixation and precipitin tests were done on the hamster serums, precipitin tests only were reported because of the consistently strong anticomplementary action of many of the serums from infected hamsters. Both precipitation and complement-fixation tests were carried out on the guinea pig serums.

In the precipitin tests, a filtrate from the growth of the mycelium in an asparagine broth was used as the antigen (Salvin and Hottle, 1948; Smith, 1943). Equal quantities of antiserum and dilutions of antigen were drawn into 3-inch lengths of capillary tubing, incubated for 2 hours at 37 C, and refrigerated for 36 hours at 3 to 5 C.

In the complement-fixation tests, a saline suspension of the cells of the yeastlike phase, killed by heating at 56 C for 1 hour, was used as the antigen. The method employed was that of Bengtson (1945), in which falling dilutions of serum, 2 units of complement, and an optimal dilution of antigen were mixed and incubated at 37 C for 1 hour before the addition of sheep cells sensitized by 2 units of hemolysin. Tests were then incubated for an additional hour at 37 C. One hundred percent end points were used throughout.

RESULTS

Cultural studies.—The number of mice from which the fungus could be recovered greatly increased when the inoculum consisted of cells suspended in 2.5% mucin instead of cells in 0.85% saline (table 1). At the dosages used, about 2.5 times as many mice injected with an inoculum suspended in mucin yielded positive cultures, as compared to mice injected with cells suspended in saline (table 1). Seven of 97 mice (7%) injected intraperitoneally with 10 cells in saline yielded cultures of *H. capsulatum*, while positive results were obtained from 46 of 97 mice (47 %) given the same number of cells in mucin. This 7-fold increase in the number of mice yielding positive cultures after injection with 10 cells contained in mucin compared to a 4-fold increase and a less than

* Obtained from the Alpine Caviary, Cheyenne, Wyoming.

† Determined by actual count in a Levy haemocytometer.

‡ This medium consisted of 51 g/L Difco cystine heart agar and 20 g/L Difco bacto-hemoglobin.

TABLE 1.—*Number of 40-day-old Swiss white male mice from one or more of whose tissues* H. capsulatum *was recovered on Sabouraud's agar after incubation at room temperature for 28 days.**

Time (in weeks) after inoculation	Number of cells inoculated into mice							
	10 in:		100 in:		1000 in:		Total	
	Saline	Mucin	Saline	Mucin	Saline	Mucin	Saline	Mucin
1	0/15†	1/15	0/15	1/15	3/15	4/15	3/45	6/45
2	0/20	16/20	0/10	2/10	5/20	16/20	5/50	34/50
4	2/13	6/13	0/8	4/8	5/13	7/13	7/34	17/34
6	0/16	8/16	0/8	4/8	8/16	5/16	8/40	17/40
8	1/18	10/18	4/8	4/8	1/18	8/18	6/44	22/44
11	4/10	1/10			8/10	5/10	12/20	6/20
14	0/5	4/5			1/5	2/5	1/10	6/10
Total	7/97	46/97	4/49	15/49	31/97	47/97	42/243	108/243
Percentage	7	47	8	31	32	48	17	45

* These data represent the results of two separate experiments.
† Numerator indicates number of animals from whose tissues *H. capsulatum* was demonstrated by culture; denominator indicates the total number of animals sacrificed and cultured.

2-fold increase when 100 or 1000 cells were employed suggests that the lower the number of cells in the inoculum, the greater is the effect of the mucin in mice.

The number of positive cultures obtained from the tissues of mice was low, *H. capsulatum* having been isolated from only 213 of 1668 tissues cultured (13%). Of the 4 tissues examined, the spleen yielded the greatest number of positive cultures, namely 107 (50%). Only 28 blood clots from the 486 mice produced growth of the fungus on culture, and 23 of these were from mice which had been inoculated with cells suspended in mucin.

The number of positive cultures from hamster tissues, in contrast to those from mice, was high (table 2), the fungus having been recovered from 142 of 171 animals (83%) and from 322 of 684 tissue specimens (47%). In addition to the animals mentioned in table 2, from each of which 4 tissues were available for culture, there were other hamsters from which all these tissues were not suitable for study. Of the 29 hamsters from which the fungus was not isolated by cultural techniques, 21 were animals inoculated with 10 cells of the yeastlike phase and sacrificed 2 weeks after injection. The number of positive cultures obtained showed little change during the period of the experiment, with the exception of the number of isolations made from blood clots. These isolations increased from 8 of 44 during the second week to 21 of 44 during the sixth week. There was no distinct difference in the number of isolations made from tissues of hamsters inoculated with cells in saline and those inoculated with cells in mucin.

The number of hamsters from which the fungus was isolated was about the same in the groups that were inoculated with either 10 or 1000 yeastlike cells. For example, during the second week postinjection, 16 of 22 hamsters that had been inoculated with 10 cells had at least one tissue from which *H. capsulatum* was isolated, whereas 22 of 22 that had been inoculated with 1000 cells were culturally positive. During the sixth week, 14 of 22 animals that had been injected with 10 cells had at least one tissue positive by culture, while 17 of 22 of those inoculated with 1000 cells were positive by culture. During the eleventh week, 6 of 7 animals injected with 10 cells had culturally positive tissues, while 11 of 12 animals inoculated with 1000 cells had infected tissues.

Hamsters obtained from Bezanson's Hamstery* acted in a similar manner as

* Temple City, California.

TABLE 2.—*Number of hamster tissues from which* H. capsulatum *was recovered on Sabouraud's agar after incubation for 28 days at room temperature.**

Time (in weeks) after inoculation	Spleen		Kidney		Liver		Blood clot		Total	
	Number	Percentage	Number	Percentage	Number	Percentage	Number	Percentage	Number	Percentage
2	34/44†	77	33/44	75	26/44	59	8/44	18	111/176	63
4	24/44	55	29/44	66	18/44	41	8/44	18	79/176	45
6	21/44	48	15/44	34	13/44	30	21/44	48	60/176	34
8	22/39	56	16/39	41	13/39	33	21/39	54	72/156	46
Total	101/171	59	93/171	54	70/171	41	58/171	34	322/684	47

* These data represent the results of two separate experiments.
† Numerator indicates number of tissues from which *H. capsulatum* was cultured; denominator indicates the total number of tissues cultured.

those used in the previous experiment. Twenty-five hamsters from each strain were inoculated intraperitoneally with 10 to 1000 cells and sacrificed 2 and 4 weeks later; tissues from each strain produced equal numbers of fungi on culture. Twenty-two of 25 hamsters of each strain yielded *H. capsulatum*.

The results of culturing the tissues of guinea pigs previously injected intraperitoneally with 10 or 1000 cells in saline or in mucin were different from those obtained from mice and hamsters. No great differences were noted in the extent of recovery of the fungus from the tissues when the yeastlike cells were suspended in mucin instead of saline prior to injection. The fungus was isolated from many more guinea pigs (45 of 70, or 64%) inoculated with 1000 cells than from those inoculated with 10 cells (14 of 70, or 20%). (See table 3.) Positive blood clots were found in only 3 of the 59 animals whose other tissues showed the fungus on culture. There was a marked reduction, over a period of time, in the number of positive cultures. For example, in animals that had been inoculated with 1000 cells in saline, when the numbers of cultures from the spleen, liver, and kidney were added together per 5 guinea pig group, 14 of 15 tissues were positive during the 2nd week postinjection, 9 of 15 during the 4th week, 3 of 15 during the 6th week, 5 of 15 during the 8th week, 4 of 15 during the 11th week, 3 of 15 during the 14th week, and none of 15 during the 16th week. Twenty-one of 25 animals that had at least one tissue from which the fungus was recovered had positive spleens. On the 40 tissues (spleen, liver, kidney, and blood clot) that were positive culturally, 21 (53%) were spleens.

One hundred and fifty additional guinea pigs were inoculated intraperitoneally with either 10, 100, 1000, 10,000, or 1,000,000 cells of the yeastlike phase in physiologic saline. Five animals in each group were bled and sacrificed every 2 weeks; the liver, spleen, kidney, and clot were cultured on Sabouraud's agar. The maximum number of isolations was made during the second week after inoculation, after which time there was a gradual decline in the number of positive cultures (table 4). Of the 67 animals that had been

TABLE 3.—*Number of guinea pigs from whose tissues* H. capsulatum *was recovered on Sabouraud's agar after incubation at room temperature for 28 days.*

Time (in weeks) after inoculation	10 in:		1000 in:		Total	
	Saline	Mucin	Saline	Mucin	Saline	Mucin
2	0/5*	1/5	5/5	5/5	5/10	6/10
4	3/5	2/5	5/5	4/5	8/10	6/10
6	2/5	0/5	3/5	4/5	5/10	4/10
8	2/5	0/5	5/5	4/5	7/10	4/10
11	1/5	0/5	4/5	2/5	5/10	2/10
13	0/5	2/5	3/5	1/5	3/10	3/10
16	0/5	1/5	0/5	0/5	0/10	1/10
Total	8/35	6/35	25/35	20/35	33/70	26/70
Percentage	(23)	(17)	(71)	(57)	(47)	(37)

* Numerator indicates number of animals from whose tissues *H. capsulatum* was demonstrated by culture; denominator indicates the total number of animals sacrificed and cultured.

sacrificed and found to have *H. capsulatum* in one or more of their tissues, 60 had culturally positive spleens, and in 32 only the spleens yielded the fungus on culture. During the second week postinoculation blood clots from all 5 of the guinea pigs in the group that had been injected with a suspension of 1,000,000 cells yielded cultures. These were the only blood clots found culturally positive during the course of the experiment.

Serologic studies.—Mice whose tissues yielded *H. capsulatum* on culture were not necessarily those that formed detectable precipitins. Of 147 mice selected for study which had been inoculated with cells in saline, 9 had precipitins alone, 5 had precipitins and tissues positive in culture, and 10 had no precipitins but tissues from which the fungus was grown. Similarly, of the 147 mice selected for serologic study which had been inoculated with cells in 2.5% mucin, 15 had precipitins alone, one had precipitins and showed the presence of the fungus in culture, and 42 had no precipitins but culturally positive tissues. Thus, no apparent relation existed in a given mouse between the presence of the fungus in the tissues and the presence of precipitins.

Of the mice inoculated with cells suspended in mucin the same number had precipitins as those inoculated with cells suspended in saline. Fourteen of 147 mice inoculated with yeastlike cells suspended in saline had precipitins; 16 of 147 mice inoculated with cells in mucin had precipitins. The mice infected with cells in mucin did not have higher titers than those infected with cells in saline.

The hamsters under study also had precipitins, and their presence was more uniform than in mice. Nineteen of 44 hamsters (43%) had precipitins during the 2nd week after inoculation, 33 of 44 (75%) during the 4th week, 39 of 44 (89%) during the 6th week; 32 of 39 (82%) during the 8th week, 14 of 19 (74%) during the 11th, and 9 of 16 (56%) during the 14th week. Peak titers (up to 1:16) were also obtained during the 6th and 8th weeks. Twelve control hamsters developed no detectable precipitins.

Few of the guinea pigs developed detectable precipitins, and then only in low titers. However, complement-fixing antibodies appeared in many of the animals and frequently in high titers. In the guinea pigs that had been infected with 10 or 1000 cells in either saline or mucin, there was no difference in the rate of antibody production or in the amount of antibody between those ani mals inoculated with cells in saline and those with cells in mucin.

The higher the inoculum, the sooner the complement-fixing antibodies appeared. For example, the guinea pigs that had been injected with 10 or 100 cells of the yeastlike phase developed antibodies to a maximum titer at 11 to 13 weeks after intraperitoneal injection, whereas those animals that had been injected with 1×10^6 cells developed maximum titers about 4 to 6 weeks after inoculation. Also, the animals that had been inoculated with 1×10^6 cells produced titers as high as 1:512; those that received 10 cells intraperitoneally developed titers only as high as 1:32. With the exception of the guinea pigs that had received 1×10^6 cells as an inoculum, complement-fixing antibodies were not detected until the second bleeding (fourth week) after injection.

The animals in each of the groups, regardless of the size of the inoculum, exhibited a marked rise and fall of complement-fixing antibodies during the first three months postinjection; the rate of antibody development and the amount of antibody varied with the size

TABLE 4.—*Relationship between size of initial inoculating dose and rate of recovery of* H. capsulatum *from spleen, liver, kidney, and blood clot of guinea pigs.**

Time (in weeks) after inoculation	Size of inoculating dose (in number of cells)										Total	
	10		100		1000		10,000		1,000,000			
	Tissues	Animals	Tissues	Animals	Tissues	Animals	Tissues	Animals	Tissues	Animals	Tissues	Animals
2	7/20	4/5	10/20	5/5	5/20	4/5	5/20	4/5	11/20	5/5	38/100	22/25
4	6/20	4/5	1/20	1/5	4/20	3/5	6/20	4/5	8/20	4/5	25/100	16/25
6	1/20	1/5	2/20	2/5	4/20	3/5	4/20	4/5	9/20	5/5	20/100	15/25
8	0/20	0/5	1/20	1/5	0/20	0/5	2/20	2/5	3/20	2/5	6/100	5/25
10	0/20	0/5	1/20	1/5	0/20	0/5	2/20	2/5	4/20	4/5	7/100	7/25
12	0/20	0/5	3/20	2/5	0/20	0/5	0/20	0/5	0/20	0/5	3/100	2/25
Total	14/120	9/30	18/120	12/30	13/120	10/30	19/120	16/30	35/120	20/30	99/600	67/150

* The animals listed were about 10 days older at time of injection than those referred to in table 3.

of the inoculum. The rise and fall of antibody titer, however, was not directly related to the time when animal tissues were found to be most heavily infected with the fungus. As seen in table 4, the fungus was most readily isolated from the spleen, liver, kidney, and blood during the first 4 weeks postinoculation. Regardless of the dose (up to 1×10^6 cells intraperitoneally), by the tenth week, *H. capsulatum* was no longer readily isolated by the cultural methods employed. Yet at that time complement-fixing antibodies could be detected in 80% of the guinea pigs (table 5). Also, the percentage of guinea pigs with complement-fixing antibodies remained high after the fourth week postinoculation until the termination of the experiment, 16 weeks after injection. It therefore may be concluded that after the first 4 to 6 weeks, serologic methods offer a better means of detecting the disease in guinea pigs than cultural methods.

DISCUSSION

Previous workers (Howell, 1948; Kotcher et al, 1951) have emphasized that *H. capsulatum* can be recovered in a very high percentage of experimentally infected animals by culture of parts of the liver and spleen. Unfortunately, this statement does not always hold true. *H. capsulatum* can be recovered by culture of the liver and spleen *if* a highly susceptible animal is used, and *if* that animal is infected with a large enough dose of virulent cells. For example, 16 of 22 hamsters had positive livers, spleens or kidneys on culture two weeks after inoculation with a saline suspension of 10 yeastlike cells. Only 5 of 50 40-day-old male mice, when injected with a similar dose of organisms and sacrificed 2 weeks later, had spleens, livers or kidneys which on culture yielded *H. capsulatum*. Therefore, a minimum infectious dose (as determined by culture) in the hamsters is less than 10 cells when administered intra-

TABLE 5.—*Relationship between cultural and serologic findings in guinea pigs inoculated with 1×10^1 to 1×10^6 cells of the yeast phase of* H. capsulatum.

Time (weeks after inoculation)	Animals with at least one tissue culturally positive		Animals with complement-fixing antibodies*		Median titer	Peak titer
	Number	Percentage	Number	Percentage		
2	27/35	77	3/35	9	0	32
4	24/35	69	19/35	54	2	512
6	20/35	57	29/35	83	4	256
8	12/35	34	29/35	83	4	256
10	7/25	28	20/25	80	8	256
12	7/35	20	27/35	77	8	64
14	3/10	30	8/10	80	8	32
16	0/28	0	20/28	71	2	32

* For the purpose of this experiment, a titer of 1:2 or greater was considered positive.

peritoneally in saline, and in the mice much greater than 10 cells.

In guinea pigs, variation in the inoculating doses produced no marked differences during the second week after injection, either in the number of tissues infected or in the number of animals infected (table 4). However, as the time between the day of inoculation and the day of sacrifice increased, the animals given the higher doses tended to have greater numbers of culturally positive tissues. Thus, during the 8th and 10th weeks, 6 of 10 pigs infected with 1×10^6 cells still showed the fungus on culture, whereas 0 of 10 pigs injected with 10 cells demonstrated the fungus on culture.

At no time was the fungus consistently isolated from the blood clots of mice or guinea pigs. However, 58 of 171 hamster blood clots (34 %) did show the fungus on culture, with the majority (72%) occurring after 4 weeks postinoculation, and after the kidney, liver and spleen had been consistently positive on culture. On the contrary, the spleens of all three species of animals were the most readily infected and the most chronically infected of the tissues studied. This was especially marked in one of the guinea pig experiments in which 60 of 67 infected animals had culturally positive spleens. Of these, 32 had only the spleens positive, with neither liver, kidney nor clot showing the fungus on culture.

One of the procedures for isolating *H. capsulatum* and for assisting in the diagnosis of human histoplasmosis has involved animal inoculation with potentially infected material. Under such circumstances, the foregoing results indicate that young hamsters are preferable to young mice (Rocky Mountain Laboratory strain) or guinea pigs. However, if mice are used, the suspected material should be suspended in 2.5% mucin, not in saline.

Precipitin tests, which were positive for mice, hamsters, and guinea pigs primarily in antigen dilutions of 1:8 and less, were of little diagnostic value in mice and guinea pigs, mainly because of the large percentage of animals with no detectable precipitins. However, precipitins in hamsters were present in almost half the animals during the second week and persisted in from 56 to 89% of the animals up to the 14th week, when the experiment was terminated.

The findings in the guinea pigs on culturally positive tissues and on complement-fixing antibodies were most interesting. The highest percentage of infected animals (77%), as shown by the culturing of excised tissues, occurred during the 2nd week after injection. Thereafter, a gradual but distinct decrease in the number of culturally positive guinea pigs was observed, until by the 16th week none of the animals had infected spleens, livers, kidneys or blood clots. These results were from animals inoculated intraperitoneally with from 1×10^1 to 1×10^6 cells of the yeastlike phase. When the serums of these same guinea pigs were examined for complement-fixing antibodies, a vastly different picture presented itself. Complement-fixing antibodies, although present in low titers in 9% of the animals during the 2nd week (all these were inoculated with 1×10^6 cells), reached their peak during the 6th and 8th weeks, at which time 83% of the animals had antibodies, with titers as high as 1:256. Thereafter, there was a gradual loss in titer, although by the 16th week when the experiment was ended, 71% of the animals still had complement-fixing antibodies. Certainly then, it would seem that under the conditions of this experiment serologic techniques offer a better

means for indication of nonfatal histoplasmosis in guinea pigs than do cultural techniques.

SUMMARY

Hamsters were more susceptible than mice to intraperitoneal infection with 10 to 1000 cells of the yeastlike phase, with guinea pigs intermediate. When 2.5% mucin was used as the suspending medium, there was a lowering of the infectious dose in mice. The spleen yielded the highest percentage of positive cultures from the tissues of mice, hamsters, and guinea pigs. Guinea pigs inoculated with higher doses (1×10^6 cells) tended to have infected spleens, livers, kidneys, and blood clots over a longer period of time than those given lower doses (1×10^1 cells). The maximum number of culturally positive tissues in the guinea pigs was during the 2nd week postinoculation, after which time there was a marked decrease up to the 16th week, when none of 28 animals were culturally positive.

There was no relationship between the presence of precipitins and of culturally positive tissues in the mice. Also, the addition of mucin to the suspending medium of the inoculating dose did not increase the number of animals with precipitins nor their titers. Hamsters showed precipitins in high percentages of animals but the titers were low over extended periods of time. Guinea pigs formed very few precipitins. However, complement-fixing antibodies were quite prominent, with serum titers ranging as high as 1:512. The greatest number of animals with complement-fixing titers occurred during the 6th to 8th weeks, although by the 16th week 71% were still positive.

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ISOLATION OF HUMAN ENTERIC PATHOGENS FROM READY-TO-EAT MEATS FROM BUTCHER SHOPS IN CAIRO, EGYPT

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Food is an important vehicle for the transmission of pathogenic enteric bacteria causing gastroenteritis in man.[1–3] This is especially true in areas where sanitation standards are low and enteric disease rates high. Poor methods of waste disposal, uncontrolled fly breeding and large numbers of human carriers provide ample opportunities for unprotected foods to become contaminated with shigella and salmonella organisms.

The importance of the role of food in the transmission of these organisms in Egypt has been pointed out by several workers,[4–16] although few reports of bacteriological investigations in this field have been found. In undertaking an epidemiological study of gastroenteritis in Egypt it was felt that a bacteriological survey of common foods in the local markets would provide further evidence of the importance of this means of transmission of the causative organisms.

Since meat had often been implicated in food poisoning outbreaks here, due to salmonella and other enteric organisms, it was the first type of food to be examined in this survey. In a report published earlier,[17] it has been shown

Received for publication May 21, 1953.

The opinions and statements contained herein are the private ones of the writers and are not to be construed as official or reflecting the views of the Navy Department or the Naval Service at large.

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that 8% of the samples of fresh meat purchased from open, unscreened butcher shops in Cairo were contaminated with shigella and salmonella organisms of probable human origin.

The present report is concerned with the bacteriological findings on samples of ready-to-eat meats (sausages, meat loaves, etc.), both imported and locally produced, that were bought from representative butcher shops and provision stores in Cairo.

MATERIALS AND METHODS

Two hundred and fifty samples of ready-to-eat meat were purchased from butcher shops and provision stores in Cairo from March through July 1952. One hundred and seventy-nine of the samples came from well sanitated, modern shops, where the meats were usually kept in refrigerated display cases. Most of these samples were imported meat loaves or sausages. The remaining 71 specimens came from small, open, poorly sanitated shops that border directly onto sidewalks or streets. In general, this type of shop served a poorer class of people and sold only locally produced ready-to-eat meats, such as "basterma" and "sago."

The types of samples selected depended upon the availability. Usually 6 to 8 samples were bought from several shops in a single day. The specimens were wrapped separately in paper and brought to the laboratory shortly after purchase.

A single thin slice, of approximately 5 to 10 g, was removed from each sample. This was triturated sterilely in a mortar with a few grams of sand and 10 to 15 ml of normal saline. After grinding, the resulting broth stood for a few moments to allow the gross particles to settle. Then 5 ml were transferred to a flask containing 50 ml of tetrathionate broth (Difco). Shigella-Salmonella (SS) agar (Difco), MacConkey agar (Difco) and sheep blood agar plates were streaked with the material. Subcultures were made from the tetrathionate flasks at 24 and 48 hours onto SS and MacConkey agar plates.

All plates were incubated at 37 C for 24 hours. Suspicious colonies were transferred to triple-sugar-iron agar (Difco) slants. Cultures showing reactions on this medium suggestive of shigellae or salmonellae were tested for their biochemical properties. Those having biochemical reactions characteristic of shigellae were identified with group and type specific serums. Those having reactions typical of salmonellae were forwarded to salmonella typing centers* for complete serological identification. Other organisms isolated from the samples were identified as far as feasible.

RESULTS

As shown in table 1, human enteric pathogens were isolated from 7 (2.8%) of the 250 samples of ready-to-eat meat examined.

Two of the samples yielded 2 enteric organisms each, making a total of 9 isolations. *Shigella flexneri 2a* was recovered once; *Salmonella paratyphi A* was found twice; *S. paratyphi B* twice; *S. paratyphi B* var. *odense*, *S. schwarzengrund*, *S. ness-ziona* and *S. cairo* once each.

This was the first reported isolation of *S. paratyphi B* var. *odense*, *S. schwarzengrund*, and *S. ness-ziona* in Egypt. *S. cario*, a new salmonella type,[18] was first isolated during this survey from a slice of bologna.

TABLE 1.—*Enteric pathogens isolated from ready-to-eat meat samples.*

	Number of samples	Number of positive samples	Organisms
Head cheese	15	0	
Veal loaf	39	1	*S. paratyphi A**
			*S. paratyphi B**
Liver loaf	22	0	
Beef loaf	13	0	
Cooked ham	26	0	
Mortadello	14	0	
Bologna	24	1	*S. flexneri 2a*†
			S. cairo†
Salami	19	1	*S. paratyphi A*
Sago	34	1	*S. paratyphi B*
Basterma	44	3	*S. paratyphi B* var. *odense*
			S. schwarzengrund
			S. ness-ziona
	250	7 (2.8%)	

* Same sample.
† Same sample.

Other enteric organisms recovered from the samples are listed in table 2.

* International Salmonella Center, State Serum Institute, Copenhagen, Denmark and Bacteriology Division, U. S. Naval Medical Research Institute Bethesda, Maryland.

18. Kauffmann, F. and Floyd, T. M. 1953, A new *Salmonella* type (*S. cairo*). Acta. Path. et Microb. Scandinavica, **32**: 124.

TABLE 2.—*Incidence of other enteric organisms in ready-to-eat meat samples.*

Organism	Percent samples
E. coli	100.0
Paracolons	38.8
Alkaligenes fecalis	34.8
Proteus sp.	23.6

Escherichia coli was present on all specimens; 38.8% contained paracolon organisms, including strains of the Bethesda, Arizona and Providence groups, which are of possible pathogenicity for man; *Alkaligenes* and *Proteus* species were found on 34.8% and 23.6%, respectively.

Table 3 lists the other pathogenic organisms, of possible human origin, that were recovered. *Micrococcus pyogenes* var. *albus* was found on all the specimens. *M. pyogenes* var. *aureus*, *Micrococcus citreus* and *Gaffkya tetragena* were often isolated. Hemolytic streptococci were recovered from 10.5% and fecal streptococci from 28.9% of the samples. A similar number contained *Candida albicans*.

TABLE 3.—*Incidence of other pathogenic organisms on ready-to-eat meat samples.*

Organism	Percent samples
M. pyogenes var. *albus*	100.0
M. pyogenes var. *aureus*	36.8
M. citreus	44.7
Gaffkya tetragena	39.4
Streptococcus hemolyticus	10.5
Group D *streptococcus*	28.9
Candida albicans	28.9

Three (1.7%) of 179 samples purchased from modern, well sanitated shops and 4 (5.6%) of 71 specimens from poorly sanitated ones were contaminated with human enteric pathogens (table 4).

TABLE 4.—*Comparative number of positives from well and poorly sanitated shops.*

	Number of samples	Number of positive samples	Percent positive
Well sanitated shops	179	3	1.7
Poorly sanitated shops	71	4	5.6
	250	7	2.8

DISCUSSION

It was not surprising to find bacteriological evidence of gross contamination of the ready-to-eat meat samples. The ingredients of such loaves and sausages are not sterile at the time of manufacture and very rapid multiplication of bacteria takes place in chopped meat.[19–21] It has been shown that salting and pickling of meat does not prevent the survival of bacteria. Various strains of salmonellae have been found to survive, without loss of pathogenicity, in dry-salted sausages for 4 months and for 2 to 6 months in those kept in brine.[22] That storage under refrigeration does not destroy these organisms has been shown in experiments when salmonella organisms have been recovered from sausages kept at −14 F for 9 months.[23]

Processed meat has been one of the most commonly incriminated vehicles in food poisoning outbreaks in European countries.[24,25] However, the organisms recovered from contaminated meats in such outbreaks usually have been

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salmonella strains of animal origin.[26–35] It has been estimated that from 10 to 90% of the contamination of processed meat originated from organisms harbored by the animals themselves.[21] Recovery of shigella strains or of salmonella strains of human origin from this source has been infrequently reported.[36–39]

Although the number of strains of shigellae or salmonellae isolated during the present study was small, it was significant that 7 of the 9 were most probably of human origin. It would seem reasonable to conclude that the ready-to-eat meat samples were contaminated by flies and human carriers, which also have been implicated in the contamination of fresh meat from butcher shops in Cairo.

Meat of this type, eaten or handled without cooking, could be a source of infection from shigella, salmonella, micrococcus or streptococcus organisms.

Of epidemiological interest was the first isolation in Egypt of three salmonella types and the discovery of a new type from the samples examined during this study.

SUMMARY

1. Two hundred and fifty samples of ready-to-eat meat from Cairo butcher shops were examined bacteriologically.

2. Human enteric pathogens were recovered from 7, or 2.8%, of the samples.

3. *Shigella flexneri 2a* was isolated once; *Salmonella paratyphi A* twice, *S. paratyphi B* twice; *S. paratyphi B* var. *odense*, *S. schwarzengrund*, *S. nessziona* and *S. cairo* once each.

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FAILURE TO PRODUCE ILLNESS IN HUMAN VOLUNTEERS FED *BACILLUS CEREUS* AND *CLOSTRIDIUM PERFRINGENS*

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In some outbreaks characterized by gastrointestinal disturbances, careful bacteriological examination does not uncover any of the recognized etiological agents of food poisoning. In some of these instances the suspected food contains large numbers, and in seemingly pure culture, of microorganisms not previously proven to cause food poisoning. This type of observation is, however, only presumptive evidence and does not constitute proof that the organism found is the agent responsible for the outbreak. The definitive test of pathogenicity of such an organism is the production of a clinical syndrome similar to that in the outbreak in a sufficient number of human beings fed pure cultures of the organism.[1] A further elucidation as to whether the food poisoning is of the infection or toxin type would also be of importance. The experiments reported in the present paper are the application of this critical human feeding test to *Clostridium perfringens* and *Bacillus cereus*, two microorganisms which have been reported as causative agents of food poisoning.

The strongest evidence for considering *B. cereus* as a cause of food poisoning has been presented by Hauge.[2] In eight different outbreaks involving some 600 persons a fairly uniform clinical picture was obtained. After an incubation period of 8 to 16 hours (usually 12 to 13) there began frequent cramp-like pains in the lower part of the abdomen and around the umbilicus. This was followed by a short period of diarrhea, usually of 4 to 6 stools. Nausea was common, but vomiting less frequent. There was no fever or weakness manifested in those patients. The incriminated food was usually starch-containing food, such as dessert sauce, which was prepared a day or two in advance of the meal at which it was served. *B. cereus* was found in large numbers in the suspected food and in the corn or potato starch or sauce powder which was used in the preparation of the dessert. A different outbreak with essentially similar findings has been reported for Norway.[3]

Hauge[2] also fed *B. cereus* grown in vanilla sauce to six human volunteers, 155 to 270 ml of the sauce, containing 30 to 60×10^6 organisms per ml, being fed to each individual. Four of these persons developed symptoms of food poisoning.

Cl. perfringens was the only organism of significance isolated from chicken dishes believed to have caused gastrointestinal upsets.[4] At least 20 persons were involved, with symptoms of nausea, vomiting (rare), intestinal cramps, and invariably a pronounced diarrhea.

Received for publication June 3, 1953.
This study was aided by a grant from the United States Public Health Service.

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4. McClung, L. S. 1945, Human food poisoning due to growth of *Clostridium perfringens* (*C. welchii*) in freshly cooked chicken; preliminary note. J. Bact. **50**: 229–231.

The incubation period was 8 to 12 hours, with diarrhea continuing about 12 hours. In most cases those who were ill were able to continue normal activities.[4] Nausea and cramps were evoked in one person and nausea and emesis in four individuals in a group of six people fed 75 to 100 ml of a heated (95 C for 1 hr) filtrate of *Cl. perfringens*. The results were less regular in nine other persons fed 50 ml or less of the heated filtrate. The symptoms in these feeding experiments appeared in 45 to 80 minutes.[5] An earlier work reported no harmful results from ingesting cultures of *Cl. perfringens*.[6]

Fifteen of 33 food poisoning outbreaks in Sweden during a 2-year period have been attributed to *Cl. perfringens*.[7] The usual symptoms were diarrhea and abdominal distress after an incubation period of 10 to 12 hours. In 7 of these outbreaks the isolated strain was inoculated into new preparations of the different implicated foods of the respective outbreak. When fed to human subjects the incubation period ranged from $8\frac{1}{2}$ to 16 hours, followed by mild diarrhea as the most common symptom. The filtrates of the samples tested were not toxic. Data as to the number of organisms fed and the exact length of incubation of the inoculated samples are lacking.

PROCEDURE

Four strains of *Cl. perfringens* (683, 689, 690, 692) that had been isolated from suspected foods were obtained from Dr. L. S. McClung. The lecithinase reaction on egg yolk medium[8] and the biochemical reactions were characteristic of *Cl. perfringens*. The four strains of *B. cereus* (3, 6, 8, 9) used were isolated by Dr. O. B. Williams from cheese spreads, attempts to obtain the original strains of Hauge[2] being unsuccessful. These strains of *B. cereus* gave identical biochemical reactions to those of Hauge's strains. They were strongly lecithinase-positive,[9] strongly hemolytic, Voges Proskauer-positive, and did not ferment xylose or arabinose.

The volunteer subjects ranged in age from 21 to 45; they were male or female physicians, nurses, students and other reliable hospital personnel. In so far as possible, those with previous gastrointestinal disorders were excluded. In no case did one individual receive more than one preparation of the same organism.

Six different series of human feedings were performed, 3 with *Cl. perfringens* and 3 with *B. cereus*. Each of these series consisted of several different experiments. Each experiment was concerned with the feeding of the test sample prepared with one of the strains of the organism. Table 1 summarizes the experiments. The test samples and controls were fed as unknowns. Plate counts were made at the time of feeding except where noted.

In feeding series 1, 2, 3, and 4 the preparations were fed before breakfast and the subjects interviewed 5, 8, and 24 hours later. Blood and urine samples were taken at the 5-hour interval. In the other feeding series, the samples were fed before supper and the subjects examined 15 hours later. A white blood count with differential, blood sedimentation rate, and a urinalysis were done on each volunteer.

Feeding series 1.—An actively growing culture of *Cl. perfringens* was inoculated into a flask of veal infusion broth (0.1% agar and 0.25% glucose)[8] which was steamed and then cooled to room temperature immediately before inoculation. After 24 hours at 37 C in air, a Seitz filtrate was prepared and then heated for 1 hour at 95 C. The filtrates were mixed with 150 to 200 ml of milk before feeding.

Feeding series 2.—The entire veal infusion broth cultures of *Cl. perfringens* (see above) were

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fed immediately after mixing with 200 ml of milk. Plate counts were made in casitone agar (5% casitone, 2% agar). Intraperitoneal injection of 1 ml of unheated filtrate of strains 689 and 692 killed mice within 48 hours, while those of strains 690 and 683 were not lethal.[5] The heated filtrates were not lethal.

Feeding series 3.—Autoclaved chicken broth inoculated with *Cl. perfringens* and incubated for 30 hours at 37 C in anaerobic jars was fed.

Feeding series 4.—*B. cereus* cells grown in nutrient broth containing 0.5% glucose (strain 6) or 1% skimmed milk (strains 8 and 9) were harvested by centrifugation after 30 hours of incubation at 32 C. The cells were washed twice with phosphate buffer (*M*/15, pH 7.4) and then suspended in a small amount of buffer. Plate counts of the suspensions were made 24 hours before feeding in nutrient agar (0.5% glucose and 0.3% yeast extract). The preparations were fed with 150 ml of milk.

Feeding series 5.—Skimmed milk inoculated with *B. cereus* and incubated at room temperature (22 to 30 C) for approximately 30 hours was the test sample.

Feeding series 6.—Vanilla sauce with a corn starch base was sterilized by autoclaving at 10 pounds for 30 minutes. This vanilla sauce inoculated with *B. cereus* and incubated 30 hours at room temperature was fed.

RESULTS

The results are tabulated in table 1. The symptoms developed by the one subject fed heated filtrate of strain 683 are not those described for *Cl. perfringens* food poisoning.[4] The symptoms described after c, d, and e are not characteristic for the *B. cereus* food poisoning that has been described. One subject (b) may possibly be interpreted as a mild case of *B. cereus* food poisoning; this seems unlikely, however, since four other persons given the same washed cell preparation and five others given whole viable cultures of the same strain did not show symptoms characteristic of *B. cereus* good poisoning.

DISCUSSION

The negative results obtained in these tests are difficult to reconcile with the positive results reported by other workers.[2,5,7] The dosages and the cultural conditions in some of the experiments were very similar to those reported as giving positive results. In the case of *Cl. perfringens* the strains tested were those isolated from the foods suspected of causing food poisoning. There is the possibility that prolonged cultivation on laboratory mediums may have resulted in a loss of pathogenicity which originally may have been present. On the other hand, it should be pointed out that the incubation period reported by Cravitz and Gillmore[5] in the positive human feedings was only 40 to 80 minutes,[5] as compared to the 8 to 12 hours of the cases reported by McClung[4] and Osterling;[7] nor is the diarrhea, which was considered an important sign, mentioned in the human feeding experiments.[5] The illness of a human volunteer reported in the original paper[4] seems to have resulted from eating a sample of the food implicated in the actual outbreak, this food presumably being free of other significant organisms. Thus, this one test cannot be accepted as a valid test of the pathogenicity of *Cl. perfringens.*

It is regrettable that the strains of *B. cereus* isolated by Hauge were not available for our study. However, the results presented in this paper do not support the contention that strains of *B. cereus* are capable of provoking gastrointestinal complaints.

It has been suggested that food poisoning may be caused by organisms which are usually nonpathogenic and are harmless when ingested in small numbers but which may produce gastrointestinal symptoms when large amounts of the organisms or their growth products are ingested.[10] Beside the *Cl. perfringens* and *B. cereus* cases

10. Hobbs, B. C. 1951, Food poisoning and food hygiene. 2-The indeterminate group of food poisoning organisms. Brit. Med. Bull. 7: 167.

TABLE 1.—*Feeding of* Cl. perfringens *and* B. cereus *preparations to human volunteers.*

Feeding series and strain no.	Preparation fed	Amount and viable cells per volunteer	Number fed* / Number reactions†	Comments on reactions
Cl. perfringeus				
1: 683	Heated filtrate	100 ml	5/1a	a. Light headed and unnatural fatigue 5 hours after the morning feeding. In evening definite weakness and generalized malaise. WBC at 5 hours, 13,600.
689	Heated filtrate	100 ml	5/0	
690	Heated filtrate	100 ml	5/0	
692	Heated filtrate	135–150 ml‡	5/0	
2: 683	Whole viable culture‖	200 ml = 2.9×10^8	5/0	
689	Whole viable culture‖	200 ml = 2.12×10^9	5.0	
690	Whole viable culture‖	200 ml = 1.29×10^9	5/0	
692	Whole viable culture‖	200 ml = 5.56×10^9	5/0	
3: 690	Whole viable culture§	250 ml = 4.62×10^8	6/0	
692	Whole viable culture§	250 ml = 1.03×10^9	6/0	
B. cereus				
4: 6	Washed cells	4.50×10^8	5/0	b. Slight cramps and 2 soft stools about 12 hours after feeding. c. One loose stool 12 hours after feeding; no other complaints.
8	Washed cells	3.71×10^9	5/1b	
9	Washed cells	8.40×10^9	5/1c	
5: 3	Whole milk culture	400 ml = 2.80×10^9	5/0	d. One loose stool 1 hour after feeding; no other complaints. e. Felt "bloated" about 2 hours after feeding, followed shortly by 1 large stool; no other symptoms.
6	Whole milk culture	400 ml = 47.6×10^9	5/1d	
8	Whole milk culture	400 ml = 3.10×10^9	5/1e	
9	Whole milk culture	400 ml = 10.4×10^9	5/0	
6: 3	Whole vanilla sauce culture	250 ml = 17.0×10^9	6/0	
6	Whole vanilla sauce culture	250 ml = 5.15×10^9	6/0	

* In each feeding experiment one other volunteer given placebo control; in series 3 and 6 no control feedings.
† See comments after corresponding letters in next column; see also under *Results*.
‡ Two received 135 ml, 3 received 150 ml equivalents of original filtrate, concentrated by vacuum distillation.
‖ Grown in veal infusion broth.
§ Grown in chicken broth.

already considered, evidence has been presented recently for the pathogenicity of an *Escherichia coli* strain[11] and of an unusual type of *Cl. perfringens*,[10] both on the basis of human feeding experiments. Nausea seems much less frequent in these *Cl. perfringens* cases than in those described by McClung.[4] (The bacteriological information in Osterling's paper[7] gives no indication whether this unusual type of *Cl. perfringens* was involved.) On this basis, it is possible that the cultures used in the experiments reported in the present paper may cause symptoms when tested under some other conditions.

The possibility of a new unrecognized agent of food poisoning must be considered in every outbreak of food poisoning where the proven agents are not found; however, it should be pointed out that the presence of these proven agents may be easily overlooked. When first cultured on a laboratory medium such as veal infusion agar, *Streptococcus faecalis* may require 2 to 3 days incubation before visible colonies are produced.[12] Staphylococcus organisms may be destroyed by heat while potent enterotoxin remains.

These possibilities do not apply to the experimental feeding of pure cultures of suspected organisms or their products of growth; however, certain precautions are necessary to establish the validity of human feeding tests. The culture medium in which the pure culture of the suspected bacteria are grown for feeding tests should be sufficiently different in

11. Ferguson, W. W. and June, R. C. 1952, Experiments on feeding adult volunteers with *E. coli* 111, B4, a coliform organism associated with infant diarrhea. Am. J. Hyg. **55**: 155–169.

12. Cary, W. E., Dack, G. M. and Myers, E. 1931, An institutional outbreak of food poisoning due to a *Streptococcus*. Proc. Soc. Exper. Biol. & Med. **29**: 214–215.

origin from that of the implicated food, that is, a different preparation of the suspected type food made from different lots of ingredients or, preferably, an entirely different substrate. This would eliminate a nonmicrobial toxicity being common to the implicated food and the medium which is being used to grow the organisms for human feeding experiments. The use of control feedings of uninoculated food would also circumvent this difficulty. But even these measures may be inadequate. Unless the food sample is sterilized before its inoculation with the suspected organism and then handled as a pure culture, the growth of other contaminating organisms should be considered. The failure of the unsterilized but experimentally uninoculated control food, which must be incubated in the same manner as the test sample, to provoke symptoms may be misleading. Under these circumstances the inoculated organism may facilitate the growth of a contaminant organism which may be a food poisoning agent or the control food may not become contaminated with the same organism which may have gained entrance into the test sample. These possibilities apparently have not been considered in the human feeding experiments with *Cl. perfringens*.[7]

If the test sample fed is distinctly unpalatable, as is the case with *Cl. perfringens*, some consideration should be given that the symptoms produced may be due to this factor alone, especially those appearing soon after the ingestion of the test material. The clinical picture obtained in the feeding test should bear resemblance to that of the actual outbreak, with due cognizance of dosage. This implies that the number of organisms present in and eaten with the food suspected of causing the outbreak should be determined and compared with the dosage of the experimental feedings.

An exact description of the circumstances under which food poisoning symptoms are evoked in human volunteers may be of some assistance in work attempting to confirm previous positive results. For example, the diet of the volunteers before, during, and after ingesting the test sample may play a role. This is suggested in the illness of children caused by *Cl. perfringens*, where the symptoms indicated not a true intoxication but an active fermentation process in the intestinal tract with mechanical distention of the bowel.[1,13] Data on the age of the culture of the test sample as well as the dosage are also useful information.

SUMMARY

The food poisoning potentialities of four strains of *Clostridium perfringens* and four strains of *Bacillus cereus* were examined by feeding these organisms and/or their products of growth to human volunteers. Under the experimental conditions employed in this work these organisms seem incapable of causing the food poisoning symptoms that have been attributed to them. In view of the negative results obtained, further evidence is required to clarify the role of *Cl. perfringens* and *B. cereus* as etiological agents in food poisoning outbreaks. Some of the conditions necessary for a valid test in the experimental feeding of human volunteers are discussed.

13. Nelson, C. 1933, Flatulent diarrhea due to *Clostridium welchii*. J. Infect. Dis. **52**: 89–93.

THE EFFECT OF LEUKOPENIA (BY NITROGEN MUSTARD) AND RETICULO-ENDOTHELIAL BLOCKADE (BY THORIUM DIOXIDE) ON TUBERCULIN CUTANEOUS SENSITIVITY

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The mechanism of the production of tuberculin sensitivity has never been established with any degree of certainty. Any responsible antibody that may be directly concerned with the tuberculin reaction has never been identified by immunologic techniques. Hypersensitiveness to homologous polysaccharides in the tuberculous host is of the anaphylactic type, distinct from the tuberculin type.[1] Certain protein fractions of the tubercle bacillus may induce the formation of anaphylactic sensitization,[2] precipitins, agglutinins and complement fixing antibodies,[3] but tuberculin sensitivity cannot be transferred to a normal animal by passive transfer of tuberculous serum,[4–6] and desensitization of skin reactivity to tuberculin is not paralleled by diminution of serum antibody concentration.[7] Thus, there is no evidence that circulating serum antibodies are concerned with delayed dermal tuberculin sensitivity.

Experimental evidence has been accumulating pointing to the possibility that the antibody or factor responsible for or mediating delayed tuberculin sensitivity is a "cellular" one. Passively transferred living white cells obtained from peritoneal exudates, spleen, lymph nodes, blood and thoracic duct lymph from tuberculin-sensitive animals confer temporary positive delayed tuberculin reactivity upon a tuberculin-negative animal of the same species.[8–13] This

Received for publication June 10, 1953.

This investigation was supported in part by a research grant from the National Microbiological Institute of the National Institutes of Health, Public Health Service.

1. Enders, J. F. 1929, Anaphylactic shock with the partial antigen of the tubercle bacillus. J. Exper. Med. **50:** 777–786.
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4. Coca, A. F. and Grove, E. E. 1925, Studies in hypersensitiveness: a study of the atopic reagins. J. Immunol. **10:** 445–464.
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6. Chase, M. W. 1952, The Allergic State, in Dubos, R. J. ed., Bacterial and Mycotic Infections of Man, ed. 2, Philadelphia, Lippincott, pp. 168–221.
7. Freund and Mansfield, cited by Opie, E. L. 1936, The significance of allergy in disease. Medicine, **15:** 489–509.
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9. Cummings, M. M., Hoyt, M. and Gottshall, R. Y. 1947, Passive transfer of tuberculin sensitivity in the guinea pig. Pub. Health Rep. **62:** 994–997.
10. Kirchheimer, W. F. and Weiser, R. S. 1947, The tuberculin reaction. III. Transfer of tuberculin sensitivity with cells of tuberculous guinea pigs. Proc. Soc. Exper. Biol. & Med. **66:** 166–170.
11. Stavitzky, A. B. 1948, Passive cellular transfer of the tuberculin type of hypersensitivity. Proc. Soc. Exper. Biol. & Med. **67:** 225–227.
12. Metataxis, M. N. and Metataxis-Bühler, M. 1948, Passive transfer of local cutaneous hypersensitivity to tuberculin. Proc. Soc. Exper. Biol. & Med. **69:** 163–165.
13. Wesslén, T. 1952, Passive transfer of tuberculin hypersensitivity by viable lymphocytes from the thoracic duct. Acta. tuber. Scand. **26:** 38–54.

technique has been successfully applied to man, utilizing peripheral white blood cell suspensions.[14] This capacity appears to be limited to mononuclear cells or lymphocytes, has not been found in granulocytes,[15] and occurs only if these cells have not undergone any damage.[6] It has been suggested that these donor cells may elaborate an antibody-like material;[6,10] and Favour and his associates[16] have been able to extract from tuberculin-sensitized lymphocytes a factor, in the nature of a labile antibody, that is precipitable with the serum euglobulins.

Evidence from the above studies would then seem to indicate that the responsible antibody, or factor, concerned with tuberculin sensitivity is either intracellular, adsorbed to the surface of cells, or intimately associated with lymphocytes, cellular elements of lymphoid tissue, and mononuclear cells, and that its in vivo action depends upon the presence of intact, uninjured, live cells and cannot exist apart from them. Therefore, any relationship noted between the selective in vivo destruction or depression of formation or activity of the lymphocyte, lymphoid cell, white cellular element of the hematopoietic system, mononuclear cell, or reticuloendothelial cell and a corresponding loss, modification, or attenuation of preexisting tuberculin sensitivity may contribute further evidence for or against the suspected relationship between tuberculin sensitivity and a "cellular" type antibody. This study is concerned with this relationship in tuberculin-sensitive rabbits. Nitrogen mustard was used to affect lymphocytic and granulocytic destruction and hematopoietic depression, and thorium dioxide was employed to produce blockade and radiation of reticulo-endothelial and mononuclear phagocytic cells that play a predominant role in the histologic evolution of the tuberculin reaction in the rabbit.[6,17]

14. Lawrence, H. S. 1949, The cellular transfer of cutaneous hypersensitivity to tuberculin in man. Proc. Soc. Exper. Biol. & Med. **71**: 516–522.
15. Kirchheimer, W. F., Hess, A. R. and Spears, R. G. 1951, Attempts at passive transfer of tuberculin type of sensitivity with living granulocytes. Am. Rev. Tuberc. **64**: 516–519.
16. Miller, J. M. and Favour, C. B. 1951, The lymphocytic origin of a plasma factor responsible for hypersensitivity in vitro of tuberculin type. J. Exper. Med. **93**: 1–12.

MATERIALS AND METHODS

Albino rabbits of the New Zealand strain, weighing approximately 1.5 to 2 kg, were infected by the intradermal injection of 0.15 mg of tubercle bacilli (BCG strain).* Three weeks later they were tested to the intracutaneous injection of 0.1 cc Old Tuberculin (O.T.) 1:10. Thirty-five animals exhibited a definite moderate to marked positive erythematous, edematous and infiltrated cutaneous reaction after 48 hours at the test site. These reactive sites were measured and traced on onion skin paper and complete blood counts were done to establish normal base line hematologic values prior to any further treatment. These 35 tuberculin-sensitive rabbits were then divided into three groups (I, II, III) for further treatment described below. All animals were tested at weekly intervals thereafter to 0.1 cc O.T., 1:10, intracutaneously for the duration of the experiment. The size of each 48-hour tuberculin skin reaction was measured and traced, its intensity noted, and blood counts were repeated so that a record was kept of hematologic values and tuberculin skin tests prior to, during, and following cessation of any drug administration.

Group I.—Fifteen rabbits received nitrogen mustard (methyl bis [B-chlorethyl] amine hydrochloride),† 1 mg/kg body weight, intravenously every 4 days to affect and maintain a leukopenia. When the size and intensity of the tuberculin re-

17. Pell, P. G. H. and Hinde, I. T. 1951, The histology of the tuberculin reaction and its modification by cortisone. Brit. J. Exper. Path. **32**: 516–529.

* Furnished through the courtesy of Dr. Joseph D. Aronson, Professor of Bacteriology, Henry Phipps Institute, University of Pennsylvania.

† Supplied through the courtesy of Dr. Stephen Fromer, Merck & Co., Rahway, New Jersey.

TABLE 1.—*Summary of effects of repeated tuberculin skin tests, nitrogen mustard (HN_2), and thorotrast (ThO_2) on pre-existing tuberculin intracutaneous reactions (O.T. 1:10) in rabbits previously rendered tuberculin-positive by infection with BCG.*

Treatment	Number of animals	Effect on tuberculin skin reaction				
		Size	Intensity	Number of animals affected	Week observed	Return to normal
None	10	—	—	10	1–8	No
HN_2	15	— — 4–5 mm — — 10–16 mm	— —	15	1 (1) 2 (7) 3 (7)	No
ThO_2	10	0	+	10	1	No

— Diminished.
+ Increased.
0 No change.

action was maintained for 4 successive weeks nitrogen mustard administration was stopped and tuberculin testing continued until the skin test had returned to its previous degree of reactivity or was maintained at the same level for 4 successive weekly tests following the return to normal hematologic values.

Group II.—Ten rabbits received 1 dose of thorium dioxide (thorotrast, 25% colloidal ThO_2 in 20% dextrin), 2.5 cc/kg body weight, intravenously. Tuberculin intracutaneous tests were done for at least the following 8 weeks.

Group III.—Ten rabbits received no further treatment or drug administration. Tuberculin intracutaneous tests were repeated for at least the following 8 weeks.

RESULTS

Group I (nitrogen mustard).—A leukopenia of 2000 to 4000 white blood cells /cu mm and characterized by neutropenia and lymphocytopenia was established and maintained. There was only a slight associated depression of red cells. A marked depression in the intensity and size of the 48-hour delayed tuberculin skin test was observed in all animals in this group. During the period of leukopenia the tuberculin test was diminished by 10 to 16 mm in diameter in each of the animals, representing an average reduction of $\frac{1}{3}$ to $\frac{1}{2}$ of the total size of the initial skin reaction. This was seen in 1 rabbit by the 7th day of nitrogen mustard administration, in 7 by the 14th day, and reached in the remaining 7 by the 21st day. This diminished positive tuberculin skin reaction did not show any further change in size or intensity, either during the period of established leukopenia in all 15 animals or during the 4 week period following re-establishment of previous normal hematologic values after the cessation of nitrogen mustard administration in the 10 rabbits that survived the entire duration of this experimental period.

Group II (thorium dioxide).—No change was noted in the peripheral white or red blood cell counts in the 10 rabbits following the intravenous administration of thorium dioxide. There was either no change or a slight reduction in the size of the test site, but a slight to moderate increase in the degree of intensity and severity over the original skin reactions was observed in all animals after tuberculin testing repeated during the 8-week period following ThO_2 administration.

Group III (untreated).—No change occurred in peripheral white or red blood cell counts. Repeated tuberculin skin tests over an 8-week period showed a slight but gradual diminution in intensity and in size of about 4 to 5 mm in diameter less than the initial positive skin reaction in all animals.

DISCUSSION

It is recognized that such factors as heredity[5] and diet[18] may play a role in

18. Long, D. A., Miles, A. A. and Perry, W. L. M. 1951, Action of ascorbic acid on

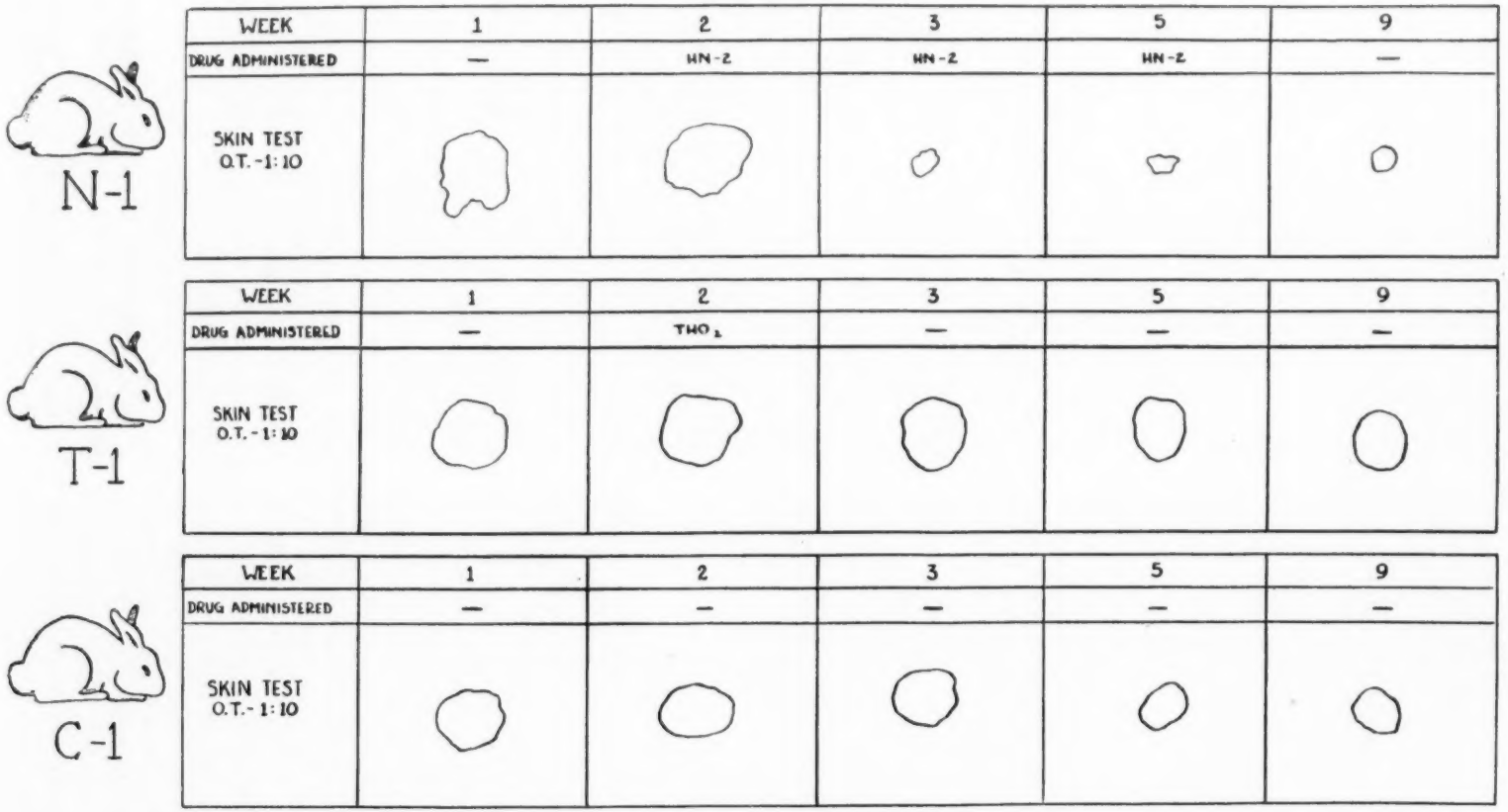

FIG. 1.—Tracings of tuberculin skin test sites 48 hours after intracutaneous injections of 0.1 cc O.T., in representative animals of each of 3 groups of rabbits receiving nitrogen mustard (N-1), thorium dioxide (T-1), and no further treatment (C-1) prior to and during the 2nd, 3rd, 5th and 9th weeks following the institution of the administration of these agents. In the case of rabbit No. N-1, the last column (9th week) also represents the 4th week after the cessation of nitrogen mustard administration.

the individual animal's reaction to infection with the tubercle bacillus and the resultant skin sensitivity reaction to tuberculin. Insofar as possible an attempt was made to control these factors by utilizing experimental animals of the same strain, all housed together under identical conditions and fed with the same stock ration.

Repeated tuberculin intracutaneous tests with O.T. over a period of 8 weeks in rabbits previously sensitized by experimentally induced infection with BCG showed the gradual development of a slight diminution in intensity and size of the positive reaction as compared with the size and intensity of earlier test reactions. This suggests either the possibility of temporary partial desensitization to tuberculin, the development of relative anergy, or an "exhaustion" of the skin reacting factors through repeated inoculations of tuberculin in these previously sensitized rabbits. However, when compared to the nontreated, partially desensitized animals, there was a much more significant marked reduction in intensity and size of the positive skin reactions in sensitized rabbits, with an associated nitrogen mustard-induced leukopenia when subjected to repeated tuberculin intracutaneous tests during the same time period.

The in vivo effects of nitrogen mustard[19] are those of fragmentation of lymphatic tissue followed by lymphoid atrophy, progressive depletion of bone marrow with cytotoxic effects on both mature and immature elements of bone marrow, fall in red blood cells, thrombocytopenia, and a reduction in the number of leukocytes with a maximum lymphocytopenia reached in 6 to 8 days and granulocytopenia in 15 to 21 days. Recovery is usually complete within the ensuing 2 to 3 weeks after administration of the drug has been stopped.

Nitrogen mustard has been shown to exert a suppressive action on the local and generalized Schwartzman phenomenon[20–23] and a partial inhibiting effect on the development of the cutaneous response to tuberculin when administered prior to and during the initial phases of infection.[24] It is also noteworthy that X radiation[25,26] and nitrogen mustard administration,[26,27] whose type of action

tuberculin sensitivity in guinea pigs and its modification by dietary and hormonal factors. Lancet, **1**: 1085–1088.

19. Wintrobe, M. M. 1951, Clinical Hematology, ed. 3, Philadelphia, Lea & Febiger, pp. 920–923.
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23. Thomas, L. and Good, R. A. 1952, The effect of cortisone on the Shwartzman reaction. The production of lesions resembling the dermal and generalized Shwartzman reactions by a single injection of bacterial toxin in cortisone-treated rabbits. J. Exper. Med. **95**: 409–428.
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26. Schwab, L., Moll, F. C., Hall, T., Brean, H., Hawn, C. V. Z. and Janeway, C. A. 1950, Experimental hypersensitivity in the rabbit. Effect of inhibition of antibody formation by X-radiation and nitrogen mustards on the histologic and serologic sequences, and on the behavior of serum complement following single large injections of foreign proteins. J. Exper. Med. **91**: 505–526.
27. Bukantz, S. C., Dammin, G. A., Wilson,

on cells resembles the effect of roentgen rays,[19] both inhibit the development of antibody formation, the Arthus phenomenon, and cardiovascular lesions of hypersensitivity. However, in these last two instances, its effect has been attributed to a suppression of formation of circulating antibody due to a quantitative reduction in antibody-producing cells. It is of interest that marked diminution in intensity and size, but not complete inhibition, of the tuberculin reaction in nitrogen mustard-treated rabbits resembles the results of the effect of ionizing X radiation on the lesions of experimentally produced contact dermatitis to chemicals,[28] in that both these manifestations of hypersensitivity are thought to be mediated by similar cellular immunologic mechanisms.

A true explanation for the findings in this study is not apparent and is a matter only for speculation. Since inhibition of the tuberculin reaction did not occur until the 14th to 21st day of an established leukopenia, this suggests that it may not have been related to immediate cytotoxic effect on mature white cellular elements, whose life span has been estimated at 13.2 days,[29] but perhaps rather through depression of hematopoiesis. If an antibody or factor responsible for tuberculin sensitivity is intimately associated with the leukocyte, the fact that it apparently was active in vivo during an induced leukopenia for the first 14 to 21 days, representing a time period equal to or exceeding the life span of a leukocyte, suggests that: (1) it might be active for 14 to 21 days after its liberation from a degenerating leukocyte; (2) its activity depends upon the presence of the circulating mature leukocyte, and when these had been completely destroyed or had disappeared from the circulation after 13.2 days, its activity was partially depressed in proportion to the depression exerted on the formation of new cells. The fact that the former degree of cutaneous sensitivity to tuberculin did not return even 4 weeks after the re-establishment of normal, hematologic values suggests that after mature cells had been destroyed this cellular antibody or factor once liberated could not be fully retained by subnormal numbers of leukocytes or by immature cells. Since this tuberculin sensitizing antibody has been thought to be associated with the lymphocyte[6] and not the granulocyte,[15] it is especially noteworthy that the first sign of suppression of the tuberculin reaction occurred during the time of reported maximum granulocytopenia (15 to 21 days) rather than that of maximum lymphocytopenia (5 to 8 days), unless this can be explained on the basis of a progressing cumulative action on all existing lymphocytes through repeated administration of nitrogen mustard.

The suppressive response of tuberculin sensitivity in rabbits to nitrogen mustard resembles their similar but only temporary response to the administration of cortisone,[17,30,31] whose action

K. S., Johnson, M. C. and Alexander, H. L. 1949, Inhibitory effect of nitrogen mustard (bis beta-chlorethyl amine) on lesions of experimental serum hypersensitiveness. Proc. Soc. Exper. Biol. & Med. **72**: 21–26.

28. Cohen, S. G., Mayer, J. D. and Criep, L. H. 1951, The effect of X-radiation on experimentally produced cutaneous sensitivity. J. Invest. Derm. **16**: 91–96.
29. Kline, D. L. and Cliffton, E. E. 1952, Measurement of white blood cells with radioactive phosphorus. Fed. Proc. **11**: 84.
30. Harris, S. and Harris, T. N. 1950, Effect of cortisone on some reactions of hypersensitivity in laboratory animals. Proc. Soc. Exper. Biol. & Med. **74**: 186–189.
31. Lurrie, M. B., Zappasodi, P., Dannenberg, A. M. and Swartz, I. B. 1951, Constitutional factors in resistance to infection: the effect of cortisone on the pathogenesis of tuberculosis. Science, **113**: 234–237.

also affects a lymphocytolysis. This is noteworthy in that "desensitization" in tuberculosis has been viewed as possibly resulting from a loss of effective circulating lymphocytes, whether due to specific desensitization with tuberculin or treatment with adrenal hormones,[6] although Stoerk[32] feels that in this latter regard it may be related to an increased degradation of antibody protein by cortisone. On the other hand, the possibility must also be considered that the suppression of the tuberculin reaction may have been related to an actual lack of available circulating and infiltrating tissue granulocytes, lymphocytes and mononuclear cells of haemic origin required for the histologic evolution of the cutaneous lesion. However, the fact that inhibition of the reaction did not occur during the first stage of leukopenia and remained suppressed even after the re-establishment of normal hematologic values is against this. The question also arises as to the possibility of the operation of similar mechanisms resulting in depression of the tuberculin reaction both in rabbits with induced leukopenia and human patients with certain intercurrent febrile diseases and cachectic or debilitating states. However, in these latter conditions the tuberculin anergy is transient and previous reactivity reappears when the nonspecific repressive factors disappear.

Thorium dioxide is a long lived natural radioactive material that is readily taken up by phagocytic cells and cells of the reticulo-endothelial system.[33] The deleterious effects of this material are due to the α particles emitted from mesothorium, a disintegration product of thorium. Alpha rays are the most destructive rays biologically and the ionization due to α radiation is locally intense. Administration of thorium dioxide results in an initial leukopenia of short duration with relative lymphocytosis,[34] cellular destruction and areas of necrosis in lymph nodes, and loss of lymphoid structure.[34–36] The elimination of thorotrast is insignificant and it remains indefinitely in the cells of the reticulo-endothelial system,[34] thus not only causing a blocking action on those cells but also subjecting these cellular elements to a continuous ionizing radiation effect. By these actions thorotrast has been found capable of abolishing tolerance to pyrogens[37] and natural and acquired immunity and resistance to the Shwartzman phenomenon,[38–39] but failed to inhibit anaphylaxis in guinea pigs.[40]

The α ray activity of 2.5 cc of thorotrast is equivalent to 0.1 μg of radium,[34]

32. Stoerk, H. C. 1950, Inhibition of the tuberculin reaction by cortisone in vaccinated guinea pigs. Fed. Proc. **9**: 345.
33. Maxfield, M. A. and Mortenson, O. A. 1941, Use of the radioactive properties of thorium for a quantitative study of phagocytosis. J. App. Physics, **12**: 197–202.
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39. Bennett, J. L., Jr. and Chiff, E. E. 1951, Studies in acquired resistance to the Shwatzman phenomenon. J. Clin. Investigation, **30**: 628–629.
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so that each animal in this experiment was subjected to that amount per kg of body weight. However, the administration of thorium dioxide in this dose did not suppress the tuberculin reaction. This suggests that the activity of the antibody or factor concerned with tuberculin cutaneous sensitivity does not depend upon an intimate association with the unimpaired activity or survival of the above mentioned types of cells affected by this agent. The increase in the degree of intensity and severity of the tissue reaction to O.T. in tuberculin-sensitive animals following the administration of thorium dioxide is noteworthy in that proliferation and differentiation of the infiltrating monocytes, local histiocytes and undifferentiated mesenchymal cells play a prominent role in the histologic evolution of the tuberculin reaction,[17] and it is the phagocytic and reticulo-endothelial cells that are chiefly affected by the intravenous administration of this agent. It is of interest that the type of cellular response affected by nitrogen mustard administration may be more inhibitory on the formation of the tuberculin reaction than that resulting from thorium dioxide in that cellular components of this lesion are derived both from infiltration of cells of haemic origin and by hyperplasia and proliferation of indigenous cells of the local reticulo-endothelial.[17] However the significance of this, by microscopic histologic study of the cutaneous tuberculin reaction, correlated with the administration of nitrogen mustard and thorium dioxide, is a matter for further investigation.

SUMMARY

1. Thirty-five albino rabbits rendered tuberculin sensitive by intradermal infection with BCG vaccine were tested intracutaneously with Old Tuberculin at weekly intervals thereafter.

2. Ten of these rabbits received no additional treatment and showed a subsequent and gradual development of slight diminution in intensity and size of the positive cutaneous reacting sites.

3. Nitrogen mustard was administered to a series of 15 of the rabbits during the period of tuberculin testing. By the 14th to 21st day of the induced leukopenia there was a marked diminution in size and intensity of the tuberculin skin reaction. In 10 animals that survived, tuberculin cutaneous sensitivity remained diminished despite a return to normal hematologic values.

4. Ten rabbits received thorium dioxide. There was either no change or a slight diminution in size of the tuberculin skin test site, but a slight to moderate increase in the degree of intensity of the cutaneous reaction was noted.

5. An explanation for these results is not apparent. The possible significance of these findings are discussed, regarding: (1) the relationships of tuberculin sensitivity, the responsible cellular "antibody" or "factor," and the leukocyte affected by nitrogen mustard; and (2) the relationship of the histologic cellular components of the tuberculin skin test and infiltrating phagocytic cells and indigenous cells of the local reticulo-endothelial system affected by thorium dioxide.

THE CAUSE OF INCREASED SUSCEPTIBILITY OF MICE TO AVIRULENT *PASTEURELLA PESTIS* AFTER EXPOSURE TO A SUBLETHAL DOSE OF X IRRADIATION

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Schechmeister and Bond[1] have reported that mice exposed to a sublethal dose of X irradiation were killed by a subsequent injection of living or heat-killed avirulent *Pasteurella pestis* or by a Seitz filtrate of a broth culture of the organisms, but that nonirradiated mice were not affected or were affected only to a lesser degree. Earlier, Jawetz and Meyer[2] had shown that large numbers of avirulent *Past. pestis* at first invaded the host rapidly, but were removed by the normal clearing mechanisms. They concluded that when death occurred, it was due primarily to an intoxication resulting from the plague endotoxin initially present in the inoculum, whereas death occurring after the injection of a small nontoxic dose of virulent organisms was a test of susceptibility to infection and not to intoxication.

The recent accumulation of reports on increased susceptibility of X-irradiated animals to infection[1,3–6] and the claim of some workers[7–9] that living avirulent *Past. pestis* is the vaccine of choice in plague prophylaxis pointed to the importance of determining whether such increased susceptibility was due to the growth and multiplication of the organism in the host tissues or whether

Received for publication June 20, 1953.

This work was supported in part by the office of Naval Research, Department of the Navy.

The opinions or assertions contained herein are those of the authors and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

The authors wish to thank Dr. S. S. Elberg, Professor of Bacteriology, University of California, Berkeley, California and Dr. K. F. Meyer, Director, The George William Hooper Foundation, University of California Medical Center, San Francisco, California for their continued interest and suggestions.

Appreciation is also expressed to Mr. H. M. S. Watkins, Naval Biological Laboratories, Oakland California for his generous gift of Namru strain of mice used in the preliminary part of this work.

* HMl USN.

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3. Schechmeister, I. L., Bond, V. P. and Swift, M. N. 1952, The susceptibility of irradiated mice to infection as a function of post-irradiation time. J. Immunol. **68**: 87–95.
4. Bond, V. P., Shechmeister, I. L., Swift, M. N. and Fishler, M. C. 1952, The effects of X irradiation on a naturally occurring endemic infection. J. Infect. Dis. **91**: 26–32.
5. Kaplan, H. S., Speck, R. S. and Jawetz, E. 1952, Impairment of antimicrobical defenses following total body irradiation of mice. J. Lab. & Clin. Med. **40**: 682–691.
6. Marston, R. Q., Gonshery, L., Alderman, I. M. and Smith, W. W. 1953, Experimental infection and streptomycin treatment in irradiated mice. Am. J. Physiol. **172**: 365–371.
7. Girard, G. and Robic, J. 1936, La vaccination de l'homme contre la peste au moyen de bacilles vivants (virus vaccin E. V.). Son application a Madagascar. Bull. Off. Int. Hyg. Publ. **28**: 1078–1087.
8. Grasset, E. 1942, Plague immunization with live vaccine in South Africa. Trans. Roy. Soc. Trop. Med. & Hyg. **35**: 203–211.
9. Otten, L. 1941, A live plague vaccine and the results. Mededeel. Dienst Volksgezondheidt Nederland Indie. **30**: 97.

X rays increased the susceptibility of the mouse to plague endotoxin.

Two methods for determining the correct hypothesis were suggested by recent work on the antigenic structure of *Past. Pestis.* Baker et al[10,11] have shown that several antigenic fractions can be separated from a water-soluble extract of acetone-dried *Past. pestis.* One of these, fraction 1A, was found to protect the mouse, rat and monkey against infection with the organism, but to induce no protection against the plague toxin. Fraction II, the endotoxin, was found to stimulate antitoxic immunity but not antibacterial immunity. Hence, by using the two antigens as immunizing agents, one should be able to determine whether death following injection of avirulent cells was caused by the growth of the organisms, or by the endotoxin initially present.

The second method was based on the findings of Espana[12] that the plague endotoxin is detoxified by 0.5% formalin without alteration of its antigenic structure. Heating the toxin at 56 C for 1 to 4 hours, however, changes neither the toxicity nor the antigenicity. Therefore, if animals were injected with heat-killed organisms in a sufficiently large dose, death due to intoxication would be expected to follow, provided the heat resistance of the cell-bound toxin is the same as that of the free toxin. On the other hand, if the animals were injected with a formalin-killed suspension, no deaths should result.

10. Baker, E. E., Somer, H., Foster, L. E., Meyer, E. and Meyer, K. F. 1947, Antigenic structure of *Pasteurella pestis* and isolation of a crystalline antigen. Proc. Soc. Exper. Biol. & Med. **64**: 139–141.
11. Baker, E. E., Somer, H., Foster, L. E., Meyer, E. and Meyer, K. F. 1952, Studies on immunization against plague. I. The isolation and characterization of the soluble antigen of *Pasteurella pestis*. J. Immunol. **68**: 131–146.
12. Espana, E. M. 1949, The antigens of *Pasteurella pestis* with special reference to the toxin. Thesis, University of California, Berkeley.

METHODS AND MATERIALS

White mice of the Webster strain, weighing 18 to 20 g, were employed throughout this study. In all experiments each experimental and control group consisted of 10 male and 10 female mice.

All test mice received 350 r whole body X radiation, a sublethal dose. Each group of 20 mice was exposed in plastic containers rotated on a turntable to insure uniform irradiation. Usually mice from each experimental and control group were exposed simultaneously. The X-ray factors were: 250 KVP, 15 ma, 0.5 mm Cu and 1.0 mm Al filters. The distance from target to skin was 63.5 cm and the output in air was 55 r per minute.

The avirulent strain, A1122, of *Past. pestis* was obtained from the Naval Biological Laboratories. This was the same source used by Shechmeister and Bond.[1] The suspensions for injection were prepared by growing the organism in flasks of Difco brain heart infusion medium shaken at room temperature for 24 hours. The organisms were collected by centrifugation, resuspended in phosphate buffer-saline at pH 7.2 and refrigerated at 5 C until used. Viable cell counts were made, using 3% sheep blood agar plates incubated at 37 C for 48 hours. After determining the number of organisms, the suspension was diluted to the desired concentration in buffered saline. Plate counts were made of the final suspensions at the time of injection into the animals.

The mice were injected subcutaneously with 0.2 ml of the suspension 6 days after exposure to X ray and were observed for 30 days after injection.

In those experiments in which post-mortem cultures were prepared, the spleen and, in some cases, other tissues showing gross pathological changes were ground with sterile sand and saline. Aliquots of the tissue homogenates were streaked on blood agar, and on replicate plates which contained 1:800 concentration of chloral hydrate to inhibit the spread of *Proteus*. Difco brain heart infusion and thioglycollate broths were also used. All cultures were incubated 96 hours before being considered negative. Final identification of *Past. pestis* was made by agglutination with antiplague serum prepared against strain A1122 and by lysis with plague bacteriophage, using the technique described by Gunnison, Larson and Lazarus.[13]

13. Gunnison, J. B., Larson, A. and Lazarus, A. S. 1951, Rapid differentiation between *Pasteurella pestis* and *Pasteurella pseudo-*

Fractions IA and II were prepared essentially by the methods described by Baker et al.[10,11] The fraction IA preparation used was that designated as fraction IA-4L by Silverman et al.[14] Fraction II was reprecipitated three times with 0.65 saturated ammonium sulfate before dialysis.

Immunization for those mice receiving fraction IA was accomplished with two subcutaneous injections of 35 μg each of alum precipitated antigen given a week apart. It has been shown previously that the 50% protective dose of this preparation consisted of 13 μg.[14] The mice receiving fraction II were given a series of five subcutaneous injections a week apart, consisting of two injections of the toxin diluted 1:10,000 (a sublethal dose), two diluted 1:1000 and one diluted 1:100. The diluent used for the toxin consisted of normal saline containing 0.1% gelatin. Eight days after the final injection of antigen, the mice were exposed to X radiation and six days later the animals were injected with varying numbers of *Past. pestis*, strain A1122. The end point of observation was the death of the animal within 30 days after challenge.

RESULTS AND DISCUSSION

The initial experiments were designed to compare the number of organisms necessary to kill X irradiated and nonirradiated mice. The response of the animals to injection of avirulent *Past. Pestis* is shown in table 1.

It can be seen that mice exposed to a sublethal dose of X rays succumbed in greater numbers than the normal mice to each dose of organisms, providing the number of organisms is within the toxic dose, but not so large as to reach the LD^{100} for nonirradiated animals. Furthermore, smaller numbers of organisms were required to kill the irradiated mice. Below a certain minimum dose, however, no significant mortality occurred in either group. This minimum dose was smaller for the X-radiated group than for the nonradiated mice, again suggesting that death was not due to multiplication of the organisms in the host but rather to the toxin injected. If the former mechanism occurred, as Jawetz and Meyer[2] have pointed out, death would eventually follow even in those animals receiving the smaller number of organisms, when the number increased sufficiently to produce an intoxication.

TABLE 1.—*Mortality in the Webster strain of mouse injected subcutaneously with varying numbers of avirulent* Past. pestis. *strain A1122, 6 days after sublethal X irradiation.*

Number of organisms injected	X-irradiated		Nonirradiated	
	Dead/ total	Percent deaths	Dead/ total	Percent deaths
3.4×10^{8}	20/20	100	20/20	100
3.4×10^{7}	17/20	85	11/20	55
1.1×10^{7}	4/20	20	0/20	0
6.8×10^{6}	2/20	10	0/20	0
4.9×10^{6}	0/20	0	0/20	0
3.4×10^{6}	2/20	10	1/20	5
3.4×10^{5}	1/20	5	1/20	5
Controls	0/20	0		

Further support of this hypothesis was obtained from the data in table 2, in which the responses of groups of irradiated and nonirradiated mice to heat-killed and formalin-killed suspensions of *Past. pestis* are compared. Heat-killed organisms were lethal for both irradiated and nonirradiated mice when given in sufficiently large doses, there being no significant difference between the two groups. The formalin-killed cells, on the other hand, were without effect on either group of mice. The few deaths obtained in these groups of animals were probably not significant. Therefore, it seems apparent that destruction of the toxin prevents the lethal effect of a large dose of avirulent *Past pestis.*

Further evidence that death is due to the toxic effects of the organism was provided in experiments in which one group of mice was immunized with fraction IA, thereby inducing an antibacterial immunity, and a second group

tuberculosis by action of bacteriophage. J. Infect. Dis. **88**: 254–255.

14. Silverman, M. S., Elberg, S. S., Meyer, K. F. and Foster, L. 1952, Studies on immunization against plague. III. Quantitative serological studies on an immunizing antigen of *Pasteurella pestis*. J. Immunol. **68**: 609–620

TABLE 2.—*Comparison of the mortality of mice injected with either heat-killed or formalin-killed suspensions of avirulent* Past. pestis *after exposure to a sublethal dose of X rays.*

Challenge dose	Heat-killed				Formalin-killed			
	X-irradiated		Nonirradiated		X-irradiated		Nonirradiated	
	Dead / Total	Percent dead	Dead / Total	Percent dead	Dead / Total	Percent dead	Dead / Total	Percent dead
1.0×10^{9}	20/20	100	19/19	100	2/20	10	0/19	0
5.0×10^{8}	18/20	90	19/20	95	1/20	5.0	0/20	0
1.0×10^{8}	11/20	55	7/20	35	1/20	5.0	0/20	0
5.0×10^{7}	5/20	25	4/20	20	1/20	5.0	0/20	0
Irradiation controls	2/15	13.3			1/20	5.0		

was injected with fraction II, thus stimulating an antitoxic immunity. Table 3 shows that the antibacterial immunity conferred no significant degree of protection on either the irradiated or nonirradiated animals. Fraction II, on the other hand, completely protected both groups of mice. It can be inferred, therefore, that death was due to the amount of toxin introduced with the initial dose of organisms injected.

Bacteriological studies at necropsy showed that *Past. pestis* could be isolated from the spleen of some of the animals as late as 12 days post infection. Recovery of *Past. pestis* was independent of exposure to X rays. The organism was isolated from some animals immunized with fraction IA but only from one animal immunized with fraction II. It is possible that a greater number of positive cultures may have been obtained if, in addition to the spleen, the regional lymph node and liver had also been cultured. In addition, in some cultures *Past. pestis* may have been overgrown by other organisms, particularly by *Proteus*, since it was not always possible to do the bacteriological examinations immediately upon death, and, in some cases, several hours may have elapsed between death and autopsy.

The isolation of avirulent *Past. pestis* from the spleen of animals injected with the organism is in agreement with the recent results of Walker et al.[15] These workers were able to isolate strain A1122 from mice for as long as 9 days after inoculation with either 10,000 or 100,000 organisms. They concluded that this strain did not multiply excessively, if at all, in the host, but merely persisted in the spleen.

The predominant organisms isolated from those animals which yielded negative cultures for *Past pestis* were *Proteus*, coliforms and *Pseudomonas*. Miller et al[16] reported that these organisms, among others, were isolated from the blood and from the spleen of animals sacrificed during the second week after exposure to 600 r or 450 r. Schechmeister and Bond[1] also reported the isolation of normal enteric bacteria from various organs cultured from mice serially sacrificed after exposure to 350 r dose of X rays and injection with avirulent *Past. pestis*. The present studies indicate that these organisms may have played a role in the death of these animals. too. However, it should be emphasized that in the work reported here all bacteriological studies were done after the death of the animals. Therefore, it is possible that these organisms

15. Walker, D. L., Foster, L. E., Chen, T. H., Larson, A. and Meyer, K. F. 1953, Studies on immunization against plague. V. Multiplication and persistence of virulent and avirulent *Pasteurella pestis* in mice and guinea pigs. J. Immunol. **70:** 245–252.

16. Miller, C. P., Hammond, C. W. and Tompkins, M. 1951, The role of infection in radiation injury. J. Lab. & Clin. Med. **38:** 331–343.

TABLE 3.—*The effect of immunization with antigenic fractions of* Past. pestis *on the mortality of mice injected with avirulent* Past. pestis *after exposure to a sublethal dose of X irradiation.*

Challenge dose	X-irradiated				Nonirradiated			
	Immune		Nonimmune		Immune		Nonimmune	
	Dead / Total	Percent deaths	Dead / Total	Percent deaths	Dead / Total	Percent deaths	Dead / Total	Percent deaths
Immunized with fraction IA								
1.05×10^{8}	9/14	64.2	11/15	73.3	10/16	66.6	6/15	40
5.3×10^{7}	10/15	66.6	5/15	33.3	0/15	0	0/15	0
1.05×10^{7}	1/20	5	2/18	11.1	0/20	0	0/20	0
Phosphate-saline	1/15	6.6	1/15	6.6	0/15	0	0/15	0
Immunized with fraction II								
1.05×10^{8}	0/20	0	13/16	81.2	0/19	0	9/20	45
5.3×10^{7}	0/18	0	5/18	27.7	0/20	0	0/20	0
1.05×10^{7}	2/18	11.1	5/18	27.7	0/20	0	1/19	5.2
Phosphate-saline	0/19	0	2/20	10.0	0/18	0	0/18	0

may have invaded the tissues during the agonal stage or after death.

In order to rule out the possibility that a selective growth of more virulent organisms from the bacterial population may have occurred in the irradiated host, 15 cultures of *Past. pestis* isolated at autopsy were screened in nonirradiated mice for increased virulence. Groups of 20 mice each were given subcutaneous injections of approximately 1×10^{7} organisms, a sublethal dose as far as the parent strain was concerned. None of the cultures tested caused the death of the nonirradiated animals, indicating that, in those cultures tested, no increased virulence had occurred.

Although the number of avirulent *Past. pestis* required to kill irradiated animals was found to be less than that necessary to kill normal animals, the amounts injected were still within the toxic range of this strain. For example, Jawetz and Meyer[2] found that the toxic dose of their cultures of *Past. pestis* strain A1122 for the mice employed was 4×10^{6} organisms, whereas for the Webster mice the LD_{50} dose of the culture used in this investigation was found to be 2.6×10^{7} for the nonirradiated animals. In the X-irradiated animals it was 7.0×10^{6} organisms.

Based on the findings that (1) organisms detoxified with formalin were not lethal and (2) that fraction II, the endotoxin, conferred immunity on the animals, while fraction IA, the antibacterial envelop antigen, did not elicit protection against avirulent *Past. pestis* when injected in large numbers, it must be concluded that the cause of death of animals infected with this organism after exposure to sublethal doses of X irradiation is due to the toxin present in the original inoculum, and is similar to the cause of death in similarly treated but nonirradiated animals. The former group, however, is more susceptible to the toxin than the latter. Until the pharmacological and physiological effects of the plague endotoxin are more clearly understood, the mechanism of increased susceptibility of X-radiated animals to the toxin cannot be explained.

The increased susceptibility to plague toxin of animals exposed to sublethal doses of X ray is not unique. Adler and Schechmeister[17] recently reported that such animals succumbed to approximately half the dose of *Clostridium*

17. Adler, F. L. and Shechmeister, I. L. 1952, Effect of sublethal total body X-irradiation on susceptibility of mice to *Clostridium septicum* toxin. Proc. Soc. Exper. Biol. & Med. **80**: 660–664.

septicum toxin required to kill nonirradiated mice. Unpublished data from our laboratory indicates that irradiated mice require less tetanus or diphtheria toxin to kill them than do nonirradiated animals. It appears, therefore, that there is a generally increased susceptibility of X-radiated animals to bacterial toxins.

The general stress of radiation injury is probably sufficient to lower the resistance of many of the susceptible tissues and organs, so that it should not be surprising that a decreased resistance not only to bacterial infection but also to the bacterial toxins occurs.

SUMMARY

1. The mechanism by which an avirulent strain of *Pasteurella pestis* induces fatality in mice exposed to sublethal doses of X ray has been investigated.

2. Formalin-killed organisms in which the endotoxin of *Past. pestis* was presumably detoxified, do not cause the death of either irradiated or nonirradiated mice when injected in comparable numbers. Heat-killed organisms, in which the endotoxin activity is preserved, were found to be lethal to both groups of animals.

3. Immunization of mice with fraction II, the plague endotoxin, followed by exposure to X rays conferred complete protection upon the animals against subsequent injection with living avirulent *Past. pestis.* Immunization with fraction IA, the antibacterial antigen, however, did not protect the animals.

4. No increased virulence could be detected among clones of *Past. pestis* isolated at necropsy.

5. It is concluded that the increased mortality in groups of animals injected with an avirulent strain of *Past. pestis* after exposure to a sublethal dose of X rays is due to increased susceptibility of the animals to the plague endotoxin.

LEPTOSPIROSIS IN SWINE CAUSED BY *LEPTOSPIRA POMONA*

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Reports from various parts of the world indicate that swine are important hosts for *Leptospira pomona*.[1–10] Evidence of infection by this organism has also been demonstrated in other species, including cattle and man.[8,10] It has been established that pigs may serve as reservoir hosts for the disease in man;[2] furthermore, there is reason to believe that an interchange of infection occurs between swine and cattle.[10,11] The disease produced in pigs by *Lept. pomona* has not yet been fully defined, nor has its significance been appraised.

The following investigations were initiated to consider certain aspects of the problem of leptospirosis in swine that seemed to be of importance: (a) a determination of the incidence of *Lept. pomona* in the swine population, (b) the characterization under controlled experimental conditions of *Lept. pomona* infection in swine, (c) the possibility that infected pigs might be a reservoir of infection for cattle.

Received for publication June 20, 1953.
Aided by grants from Armour and Co. and Chas. Pfizer and Co.

1. Schmid, G. and Giovanella, R. 1947, Schweiz. Arch. f. Tierhlk. **89**: 1–13.
2. Gsell, O. 1946, Schweiz. Med. Wschr. **76**: 237–241.
3. Babudieri, D. 1949, Rend. Inst. Super. Sanita. **12**: 937–948, in Excerpta Medica, 1950, Sect. IV, **3**: 1251.
4. Kolochine-Erber, B. and Collombier, M. 1950, Ann. Inst. Pasteur, **79**: 370–375.
5. Savino, E. and Rennella, E. 1945/48, Rev. Inst. Bact. Malbran, **13**: 66–71, in Bull. Hyg. 1950, **25**: 1246.
6. Collier, W. A. 1948, Schweiz. Med. Wschr. **78**: 508–509, in Vet. Bull. 1950, **20**: 71.
7. Gochenour, W. S., Jr., Johnston, R. V., Yager, R. H. and Gochenour, W. S. 1952, Am. J. Vet. Res. **13**: 158–160.
8. Gsell, O. 1952, Leptospirosen. Verlag Hans Uber, Bern.
9. Johnson, D. W. Cited by Gsell,[8] p. 173.
10. Bohl, E. H. and Ferguson, L. C. 1952, J.A.V.M.A. **121**: 421–428.
11. Peterson, J. E. 1951, Australian Vet. J. **27**: 40–43.

INCIDENCE OF LEPTOSPIRAL INFECTION IN SWINE

To gain some idea of the incidence in swine in the United States, serological surveys offered the best method of approach, although such surveys are subject to many variable factors, and would constitute an enormous task if statistically valid results were to be obtained. It was thought that serological studies of pigs coming to market would provide some information of the incidence, since pigs from several areas would be represented. Accordingly, serum samples were collected from pigs killed in large abattoirs in Chicago, Illinois and Denver, Colorado, and probably represented animals from certain midwestern and intermountain states. In the collection, blood samples were obtained from pigs at the time of slaughter. To make the sampling as random as possible, approximately every tenth animal was bled. In Chicago, where pigs were killed at the rate of 400 per hour, a total of 186 serums, in 2 collections a month apart, 86 in one and 100 in the other, were obtained. In Denver, the slaughter rate was 150 per hour, and 99 samples were collected.

Presence of antibodies was determined by the agglutination lysis test on all serums for *Lept. pomona* and on some for *Leptopsira icterohemorrhagiae*. In the test for *Lept. pomona*, antigen

consisted of allantoic fluids from eggs infected with Cornell T262 strain, while the antigen for *Lept. icterohemorrhagiae* consisted of organisms that had been grown in modified Schuffner's medium.[12] Antigen was mixed with an equal portion of diluted serum. After incubation for 1 hour at 37 C, each mixture was examined by darkfield microscopy and a serum considered positive if half or more of the organisms was agglutinated or lysed. In every series of tests, known negative serums were included.

These findings are presented in table 1, where it can be seen that 63 (22%) out of 285 samples were positive at dilutions of 1:200. It is interesting to note

TABLE 1.—*A serological survey of swine for* Lept. pomona.

Groups	Number tested	Number positive*	Percent
A. Chicago	86	16	19
B. Chicago	100	21	21
C. Denver	99	26	27
Total	285	63	22

* 50% agglutination-lysis at 1:200.

that similar results were obtained in all groups of animals tested. Although not shown in the table, the 21 samples in group B that were found to be positive at dilutions of 1:200 were further diluted and 13 reacted at 1:1000 and 7 at 1:10,000. Furthermore, the 100 samples in this same group were tested against *Lept. icterohemorrhagiae* and only 2 were found to be positive. The reactions, however, were only partial at the dilution tested (1:200).

Additional studies were made on aliquots of 39 serial lots of anti-hog cholera serums obtained from a large midwestern plant.* Each sample represented the bleedings of up to 175 hogs, and each lot was made up of entirely different animals. In all, 6346 different pigs were represented. The tests showed that all 39 samples were positive at dilutions of 1:200 and 32 reacted at dilutions of 1:2000.

Many of the hogs examined showed pathological changes in their kidneys that resembled the ones produced experimentally with *Lept. pomona*. Accordingly, an attempt was made to correlate the occurrence of these lesions with the serological findings for *Lept. pomona*. Kidneys of 114 of the pigs bled in Chicago were collected and examined for gross lesions. Macroscopic changes usually involving both kidneys and consisting of grayish white areas that varied in size, shape, and distribution were noted in 20 of these. Kidneys from 5 of these animals were scarred and somewhat shrunken, with fibrous tissue extending through the cortex and medulla. After noting the gross changes, a wedge of tissue extending from the cortex through the medulla to the pelvis was removed from the greater curvature of at least one kidney of each pig, and immediately placed in a vial containing 10% formalin. Tissues were in the fixative within 1 hour following the death of the pig. Histological studies were made of all kidneys collected. Sections were cut at 5 μ and stained by hematoxylin and eosin. Additional sections of certain kidneys were also silver impregnated by the modified Warthin-Starry method[13] for demonstration of leptospiras. Results are shown in table 2.

As can be seen in table 2, 25 (20%) of the 114 swine tested contained antibodies against *Lept. pomona*, whereas the kidneys of 57 revealed histopathological lesions. Of the serologically positive animals, 19 showed lesions.

12. Van Thiel, P. H. 1948, The Leptospiroses. Leiden, Universitaire Pers.

* Obtained through the courtesy of Dr. B. J. Gray, Fort Dodge Laboratories.

13. Faulkner, R. R. and Lillie, R. D. 1945, Stain Tech. **20**: 81–82.

Subjecting the data in table 2 to a Chi square analysis gave a value of 7.37 which is highly significant ($P = < 0.01$). It must be pointed out that the evaluation of this analysis was limited by a number of factors, of which one of the most important was the fact that only an extremely small proportion of each kidney was examined. The type of lesions could also have had considerable influence on the analysis. In this case,

TABLE 2.—*Correlation of* Lept. pomona *antibodies and microscopic kidney lesions found in marketed pigs.*

Serological study	Total	Number of pigs with lesions	Number of pigs without lesions
Number of pigs with antibodies	25	19	6
Number of pigs without antibodies	89	38	51
Total	114	57	57

however, 56 of the kidneys examined had varying degrees of interstitial nephritis, which is the lesion of experimental leptospirosis. Recognizing these limitations then, it is probable that a significant number of the kidney lesions[14,15] that are seen in pigs coming to slaughter are caused by leptospiral infections.

Silver staining of kidney sections revealed leptospiras in 7 of the 25 pigs that had leptospiral antibodies. Also, guinea pigs were inoculated with suspensions of kidneys from 5 that were apparently normal and from 5 that showed lesions. A strain of *Lept. pomona* was isolated from one of the kidneys that had no lesions, and, furthermore, that came from a pig that had no antibodies against this leptospira.

14. Florin, M. C. 1941, Porcine Nephritis, Thesis, Cornell University.
15. Nieberle, K. and Cohrs, P. 1949, Lehrbuch der Speziellen Pathologischen Anatomie der Haustiere, 3. Aufl. Jena, Verlag Gustav Fischer.

EXPERIMENTAL DISEASE

Production.—The swine used in this study were of mixed breeds and were obtained either from a herd of disease-free animals maintained by this laboratory or from local farms. Except for the baby pigs born to sows on experiment, the ages at the time of use ranged from 3 weeks to approximately 1 year. A few of the animals used in exposure tests had recovered from other experimental infections but, as far as could be ascertained, were free from any infection when these experiments were initiated. Pigs were fed commercial feeds made of mixed grains. All disease studies were conducted in isolation units to avoid complications by extraneous infections.

The material used for inoculation consisted of defibrinated blood pooled from two or more guinea pigs infected with a strain of *Lept. pomona*, designated "MLS," that had been maintained by serial passage in guinea pigs, since its original isolation from a dead calf showing jaundice in November, 1951. Each inoculum used was tested for the presence of infective leptospiras by inoculation of guinea pigs. Also, maintenance of virulence for calves was demonstrated by production of typical illness. Pigs were inoculated by several methods: (a) of a group of 22 pigs and 3 pregnant sows, each was inoculated subcutaneously with either 1.0 or 1.5 ml of inoculum representing 14 to 85 serial passages in guinea pigs; (b) of a group of 7 pigs, each was inoculated intranasally with 1 ml of inoculum that represented 71 and 77 passages; (c) of a group of 7 pigs, each was given 1 ml orally that represented 71 and 73 passages; and (d) a group of 10 pigs was placed in pens with others that previously had been infected.

Following inoculation, all animals were observed daily for increased temperature and other signs of illness. At

TABLE 3.—*Production of Leptospirosis in swine.*

Route of inoculation	Infection demonstrated by:		Total number of pigs infected
	Isolation and serology	Serology only	
Intranasal	7/7*	0/7	7/7
Subcutaneous	24/25	1/25	25/25
Oral	1/7	0/7	1/7
Contact	9/10	1/10	10/10

* Numerator = number of pigs infected; denominator = number of pigs used.

the time of inoculation and at various intervals thereafter, tests were made for evidence of infection by usual methods, including isolation of leptospiras from infected pigs either by cultivation in Schuffner's medium or inoculation of guinea pigs,[16] demonstration of leptospiras in infected material by darkfield microscopy and silver stains, and serological studies using the agglutination-lysis test. Urine samples from a number of pigs were studied at periodic intervals during experiments for changes in specific gravity and pH, and for the presence of albumin and casts. The results are presented in table 3.

As can be seen from table 3, all animals inoculated subcutaneously, intranasally, and by exposure became infected. In feeding experiments, however, only 1 out of 7 developed infection. It was interesting to note that the oral mucosa of this pig became lacerated while being restrained. The possibility that leptospiras entered through this wound may have complicated the method used.

Clinical features.—Following inoculation, 22 of 33 pigs showed an elevation in temperature in 7 to 13 days which ranged from 105 to 107 F and usually lasted 24 to 36 hours. Several pigs responded for a short time only and, if temperatures had not been taken twice daily, the thermal responses would not have been noticed. In general, the incubation period was related to the route of inoculation; pigs infected subcutaneously responded with an elevated temperature sooner than those inoculated by the intranasal route. During the period of elevated temperature and throughout the experiments, all except one pig remained alert and ate normally. This animal was stunted at the time of inoculation, having had transmissible gastroenteritis 6 weeks previously. Following inoculation, temperatures became subnormal and the pig died 14 days later. An autopsy examination 15 hours after death revealed an intense icterus. Post-mortem autolysis made lesions difficult to evaluate, but it was noted that the pericardial sac was thickened and distended with a serosanguineous fluid. Darkfield examination of this fluid revealed numerous active leptospiras.

Samples of urine collected either prior to inoculation or early in the infection were found to have specific gravities within the normal range of 1.025 to 1.040. These values became lower, usually less than 1.010, about 3 weeks after inoculation. In 2 of the pigs that were retained on experiment for 4 and 5 months, the specific gravity remained low throughout this period. All infected pigs also were found to have albumin in the urine, concurrent with the lowering of the specific gravity. These changes are shown in figure 1.

The pH values of the urines varied from acid to alkaline but were usually in the alkaline range. The numbers of visible leptospiras did not seem to be affected by pH, since some urines in the acid range showed numerous organisms while in others the converse occurred. It was interesting to note that urines with a pH of 7 or above usually had active leptospiras, while in those that tested below 7, the organisms appeared

16. Baker, J. A. and Little, R. B. 1948, J. Exper. Med. **88**: 295–307.

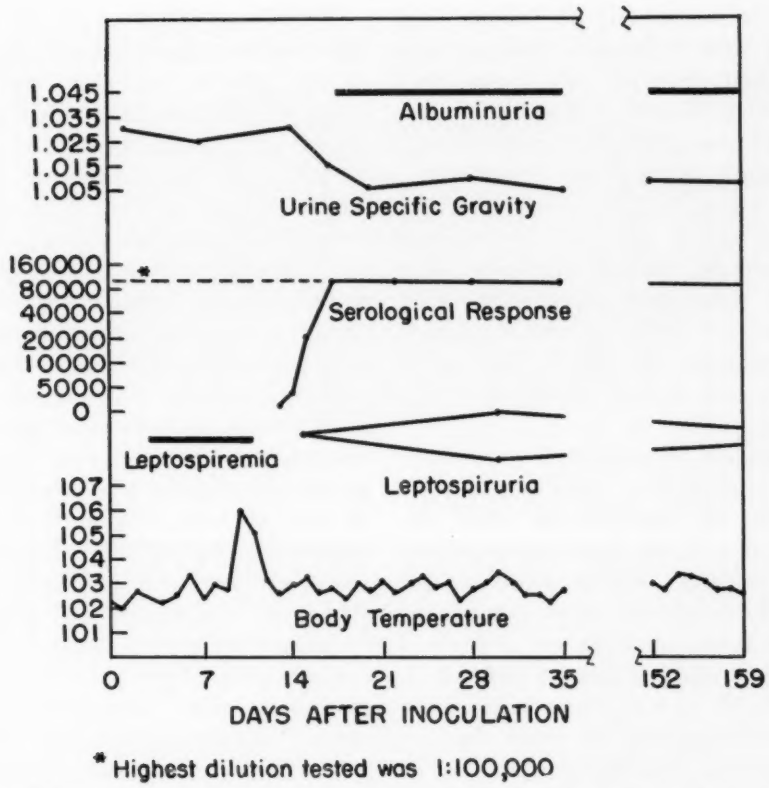


FIG. 1.—Typical clinical features shown by a pig inoculated subcutaneously with *Lept. pomona*.

inactive but were nevertheless viable, since they were still infective for guinea pigs.

In examination of sediments, casts that were predominantly cellular although sometimes hyaline were only rarely noted. They were mostly seen in the chronic cases. Many urines from chronically affected pigs contained leukocytes. On rare occasions red blood cells were noted.

The studies on the pregnant sows varied, and therefore findings are presented separately. Sow I.—Following inoculation no elevation of temperature was noted. One attempt to isolate leptospiras from the blood failed; however, the urine was shown to contain leptospiras on the day of parturition, which occurred 19 days post-inoculation. Fifteen hours after the sow farrowed, one pig appeared jaundiced, depressed, and was standing away from the others. Its serum had an icteric index of 25. The pig was destroyed and autopsy revealed icteric tissues and a liver that contained numerous gray foci. Leptospiras were isolated from tissue suspensions. The other 7 pigs of the litter showed no evidence of clinical disease. They were bled 24 hours after suckling, and at weekly intervals for a period of 1 month. Attempts to isolate leptospiras from these failed. Sow II.—This animal was inoculated 12 days before parturition. The sow showed a rise in temperature 1 day prior to farrowing. She delivered 5 pigs in a normal manner. The pigs were not allowed to suckle. Twenty-four hours after birth

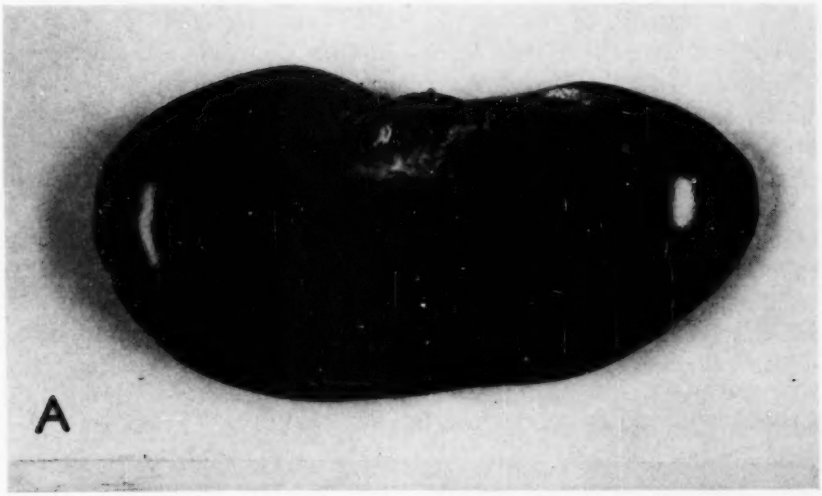
they were destroyed; no lesions were seen nor were leptospiras isolated from any of them. Sow III.—Nine pigs were farrowed by this sow 30 days after inoculation. The sow did not develop a high temperature. All pigs were apparently normal when born. Four were not allowed to suckle and, when destroyed and examined, showed no lesions. The other 5 pigs were allowed to have colostrum for 36 hours and were then bled. Attempts to isolate leptospiras from the sow as well as the pigs were negative.

Pathology.—Studies for the pathological effects of porcine leptospirosis were made on 26 experimental pigs destroyed at varying intervals after becoming infected. Seven animals were sacrificed during the period of temperature elevation, and groups of 2 or 3 each were destroyed at intervals of 1, 2, 3, 4, 6, 8, 10, and 20 weeks after either the thermal response in those that reacted, or the serological response in those that did not. Several methods of sacrificing swine were utilized. For larger hogs, electrocution was the method of choice. Smaller pigs were destroyed by chloroform, nembutal, or by stunning with a sharp blow on the skull followed by bleeding out from the jugular vein. The animals were autopsied immediately, the various organs examined and small sections of tissue from each placed in 10% formalin. Tissues were sectioned and stained with hematoxylin and eosin.

Examination of the animals destroyed during the period of temperature elevation for gross lesions revealed a few scattered petechial and ecchymotic hemorrhages in the lungs of 3 of the pigs. The livers of 2 of the same 3 were pale in color, slightly mottled, and friable. All other organs appeared normal. No gross changes were seen in the other 4 pigs destroyed during the same period.

In animals killed later in the course of the infection, the only lesions noted were in the kidneys, and these were seen in all but 3 pigs. In those with lesions, the extent and involvement of the kidneys seemed to be in proportion to the length of time the pigs had been infected. There were no macroscopic changes noted in the kidneys of any pigs sacrificed 1 to 2 weeks after temperature elevation. Three weeks afterwards, however, a few small grayish-white raised areas, 1 to 2 mm in diameter, were present (see fig. 2); when the kidneys were cut these did not extend into the cortex. The kidneys of pigs destroyed after 4 weeks and later showed an increasing number of white spots that varied in size and shape; many of these whitened areas, as they developed, appeared as depressions, which on the cut surface extended into the medulla. The capsule became difficult to strip off; following stripping the surface of some kidneys was roughened with many fine depressions. As the infection progressed, kidneys showed evidence of scarring. They appeared shrunken, and small bands of fibrous tissue were seen both on the surface and extending into the parenchyma.

Microscopic examination of sections from the lungs of animals that showed gross lesions early in the course of the infection revealed hemorrhages that varied from involvement of 1 or 2 alveoli to somewhat larger areas of the parenchyma. No lesions were seen in the lungs of the other 4 pigs. Areas of degeneration were found in the livers of all 7 pigs that were represented by nuclear changes of pyknosis and karyorrhexis and an increased granularity of the cytoplasm of hepatic cells. Occasional small focal accumulations of mononuclear leukocytes were seen in the parenchyma as well as in the perilobular interstitial tissue. There was no evidence of dissociation or disruption of



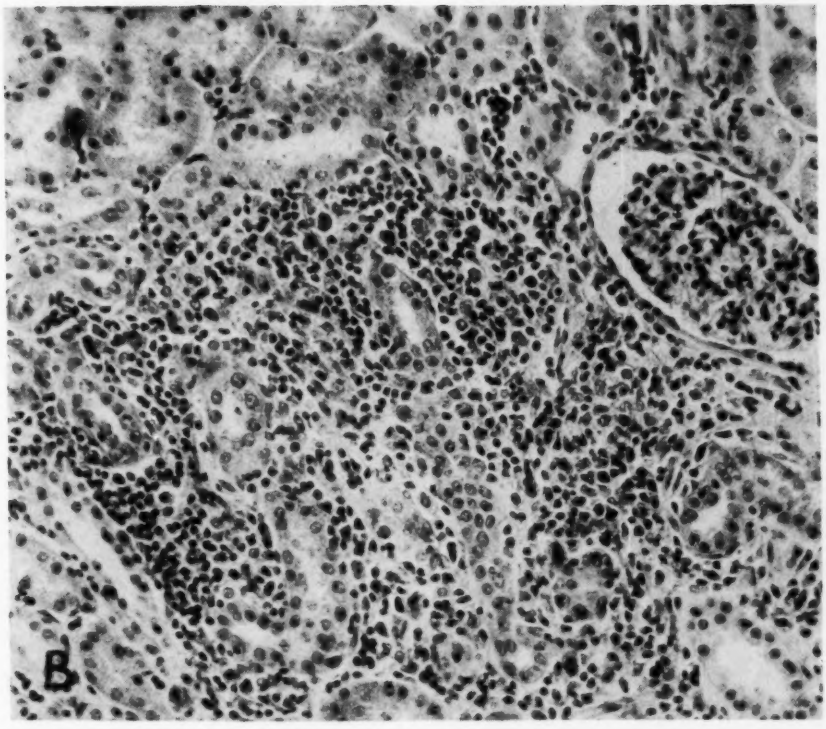


FIG. 2A (top).—Kidney from a pig inoculated with *Lept. pomona*. Note light areas that represent typical lesions.

FIG. 3B (bottom).—Section from kidney of a pig 3 weeks after inoculation with *Lept. pomona*, showing cellular infiltration. Hematoxylin and eosin stain. ×325.

hepatic cords as described for leptospirosis in other species. In the one pig that showed jaundice at birth, focal areas of necrosis were seen throughout the organ. There were mixtures of leukocytes, predominantly mononuclear but including many neutrophiles, surrounding and infiltrating these areas. In the kidneys, small areas of tubular degeneration were noted, and an occasional focal area of inflammatory reaction represented by infiltrations of mononuclear cells was seen in the interstitial tissue. There was some capillary congestion, particularly in the medulla. An occasional extravasated erythrocyte was noted. The glomeruli and vessels appeared unchanged. No lesions were found in the cerebrum, cerebellum, spinal cord, lymph nodes, spleen, adrenal glands, liver, testicles, ovaries, uterus, skeletal muscle, and cardiac muscle.

In later stages of infection microscopic lesions were found only in the kidneys. These appeared similar whatever the stage of infection and were represented predominantly by infiltrations of mononuclear cells (see fig. 3). There was no predilection for any particular area of the kidney, both cortex and medulla being involved, nor was there any restriction as to size of the infiltrating process, although most were small. The distribution was mostly focal; however, in some kidneys, the infiltrative areas were rather diffuse. Localization was perivascular, periglomerular, and mostly intertubular. The mononuclear cells were of several types. Lymphocytes, monocytes, plasma cells, and polyblasts were seen. Some cells were young and showed evidence of mitosis. Rarely, a few neutrophiles were observed. Tubules, except in the areas of infiltrations, did not seem to be involved. Cellular and hyaline casts were seen occasionally in the lumina of the tubules. In kidneys from pigs that had been infected for long periods, tubules were dilated, and occasionally hyperplastic changes were observed. In a few kidneys there was evidence of fibrous connective tissue proliferation. This change was always slight, and generally was found surrounding vessels. Occasionally, small bands of fibrous connective tissue were observed surrounding tubules. Glomeruli were not involved. This was true in spite of the fact that in some instances they were almost completely surrounded by cellular infiltrations. No vascular abnormalities were noted. It seems important to point out that leptospiras were isolated from all kidneys that had lesions.

Presence and persistence of leptospiras in the blood and urine of infected pigs.—From 17 animals inoculated either subcutaneously or intranasally, tests were made to determine if leptospiras were present in the blood. In general, most pigs were bled only once but a few were tested 2 or more times. Of the 17 pigs tested, leptospiras were found in the blood of 14, isolations having been made as early as the 3rd day and as late as the 12th day following inoculation.

To study persistence, urines from 34 infected pigs destroyed at varying intervals were tested for leptospiras and all but 3 were found excreting the organism. These 3 were also the ones that did not have kidney lesions. Leptospiras were seen in the urine of some pigs as early as the 12th day after inoculation and were isolated from all 31 pigs at the time of their death. In 2 pigs that were kept longest, the organisms persisted in the kidneys and urine of one 159 days after inoculation, and in the other, leptospiras were present in the kidney 140 days after the pig had become infected by exposure.

Usually leptospiras were readily seen by darkfield examination of unconcen-

trated urine, and the numbers of organisms frequently were as numerous as those grown in culture. The quantity observed in the urine of a given pig, however, varied from day to day and even during the same day. On occasion, several consecutive darkfield examinations revealed no organisms, only to be followed by one that did. Also, some darkfield examinations were negative but the urine was usually found to be infective for guinea pigs.

Serology.—Following inoculation with *Lept. pomona*, all animals except those destroyed too early developed antibodies against this organism. Usually antibodies became demonstrable 12 to 15 days after inoculation although in several cases titers were demonstrated as early as the 8th day. Once antibodies appeared, a high blood concentration was reached in a short time, and the level was maintained until the animal was destroyed. An occasional pig, however, never showed a titer above 1:2000. A typical serological response is presented in figure 1.

Serums from the 3 sows and their pigs were studied. Sow I showed antibodies 8 days following inoculation and developed a titer of 1:4000 that remained constant during the time on experiment. Serums from 7 of her pigs, not including the one that developed jaundice, were tested periodically until they were 30 days of age. The day after suckling, all showed approximately the same titer as the sow. This was followed by a progressive decline in antibodies during the month of testing to titers of 1:80 to 1:320. The jaundiced pig had a titer of 1:80 when destroyed. Neither sow II nor her litter developed leptospiral antibodies. Sow III, like sow I, had antibodies 8 days after inoculation, and 12 days later developed a titer of 1:64,000 that remained constant until she was destroyed. No antibodies were found in 4 of her pigs that were not allowed to suckle, whereas the 5 that received colostrum had the same titer as the sow. Only the one test was made on the latter group.

EPIDEMIOLOGY

An effort was made to demonstrate the transmissibility of *Lept. pomona* infection between swine and cattle. In one experiment, 3 pigs and 1 calf were exposed to 3 infected pigs. In a second test, 2 pigs and another calf were exposed to 2 more infected pigs. The third experiment utilized 5 pigs and 2 calves in exposure to 4 infected pigs. In a reciprocal test, 3 pigs were exposed to an infected calf. In another trial, this was repeated with 4 pigs exposed to a calf. Presence of leptospiras in the urines of infected animals was established by guinea pig inoculations. Temperatures of all animals were taken daily and observations were made for other signs of illness. At intervals throughout the experiments, blood and urine of exposed animals were checked for evidence of leptospiral infection by isolation attempts and serological studies. The results of exposure tests are shown in table 4.

As can be seen in table 4, infection occurred in all calves and pigs exposed to infected pigs. The time between exposure and demonstration of infection varied from 12 to 45 days. Pigs exposed to infected calves failed to contract infection.

TABLE 4.—*Results of exposure tests between swine and cattle infected with* Lept. pomona.

Source	Number infected	Exposed animals		Days after exposure infection demonstrated	
		Swine	Calves	Swine	Calves
Swine	3	3/3*	1/1	12–22	31
	2	2/2	1/1	16–25	18
	4	5/5	2/2	27–45	36–45
Calves	1	0/4	None		
	1	0/3	None		

* Numerator = number contracting infection by exposure; denominator = number exposed.

DISCUSSION

In studies characterizing experimental leptospirosis of swine produced by *Lept. pomona*, all animals inoculated subcutaneously, intranasally, and those exposed by contact became infected. Oral administration of leptospiras was not successful except in one instance, in which there was a complicating factor. Organisms were isolated from the blood stream as early as the 3rd day and as late as the 12th day after inoculation. After an incubation period of approximately 10 days, a transitory elevation of temperature occurred in 22 out of 33 inoculated animals. With two exceptions, pigs were free from signs of illness, appeared alert, and ate normally. In these 2 animals, the infection became fulminant and resembled the severe icteric forms of leptospirosis occasionally seen in dogs, cattle, and man.[8] These results compared with the findings of Schmid and Giovanella[1] in Switzerland, who noted that 3 out of 6 experimentally infected pigs became febrile with a concomitant inappetance and conjunctivitis approximately 3 weeks after the start of infection. These workers concluded that *Lept. pomona* infection in swine was essentially benign.

One to two days after the thermal reaction antibodies were demonstrated in the blood and leptospiras could no longer be isolated. Antibodies as tested by agglutination-lysis methods reached high concentrations in a few days, and in the pigs studied, these levels were maintained for the length of the experiment. Concurrent with the demonstration of antibodies, leptospiras were found in the urine of all but 2 pigs that were studied. The leptospiras were usually present in enormous numbers, although in several animals, relatively few were seen. One pig was shown to be shedding leptospiras 159 days after inoculation. Schmid and Giovanella[1] observed leptospiras in the urine of 2 experimental pigs approximately 1 year after infecting the animals.

There were few lesions observed in animals destroyed during the period of temperature elevation. The lungs of several pigs contained a few small hemorrhages, and in some, mild degenerative changes were noted in the livers and kidneys. In pigs sacrificed later in the course of infection, the only lesions seen were in the kidneys—the changes of interstitial nephritis. The nephritis appeared to be progressive, and although kidneys of longstanding cases were considerably involved, fibrosis was not a frequent finding. The renal aberrations of the chronic cases were reflected upon the changes in the urine. As the infection progressed, the specific gravity of the urine fell and albumin became demonstrable.

The nature of the porcine disease, then, on the basis of the evidence presented, is such that it would seem reasonable to expect the infection to be widespread in the swine population. Reports in the literature apparently substantiate this. In Switzerland, Gsell[2] reported 114 out of 193 pigs (59%) tested to be serologically positive to *Lept. pomona*. Babudieri[3] in Italy found 23 out of 73 serums examined (32%) to be positive. In France, Kolochine-Erber and Collombier[4] tested 172 serums, of which 62 (36%) contained antibodies for *Lept. pomona*. In Argentina, Savino and Rennella[5] reported 26 out of 201 (13%) to be positive. These workers isolated strains of *Lept. pomona* from the kidneys of healthy pigs. Collier[6] isolated 6 strains of *Lept. pomona* from 104 pigs on one farm in the Dutch East Indies. He also tested 64 serums from other swine and 30 (46%) were found to have antibodies for this organism. Johnson[9] in Australia tested 251 serums, with 107 (43%) reacting posi-

tively. He also procured strains of *Lept. pomona* from the kidneys of apparently normal pigs. Bohl and Ferguson[10] in the United States reported that 5 out of 280 (2%) swine samples collected at random were serologically positive. They also isolated the organism from a healthy pig.

To gain further information about the incidence of infection in this country, two approaches were utilized. First, a serological survey was made of 285 serums collected in large packing houses and 63 (22%) were found to be positive; additional studies on 39 samples of anti-hog cholera serums representing over 6000 hogs revealed that all contained leptospiral antibodies. Secondly, since experimental *Lept. pomona* infection was characterized by the pigs having interstitial nephritis, and since many normal swine coming to slaughter have similar lesions, an attempt was made to correlate positive serological findings for *Lept. pomona* with the presence of these lesions in swine slaughtered at abattoirs. The results showed that a significant number of the swine with these lesions also had evidence of leptospirosis. As an incidental finding, it was observed that 50% of the kidneys from the 114 swine studied had histopathological lesions. Although these studies suggested that many of the renal lesions present in swine are produced by leptospirosis, they also indicated that there are other causes. Furthermore, if the evidence presented here and that reported in the literature are any true indication of the incidence and nature of *Lept. pomona* infection in swine, it appears important to stress that finding evidence of leptospiral infection in a pig with an undiagnosed disease should not be the sole basis for incriminating leptospiras as the etiological agent.

Evidence that *Lept. pomona* exists in both cattle and swine suggested the possibility that these animals might serve as reservoir of infection for each other. Bohl and Ferguson[10] conjectured that this may occur since cattle and swine on the same farm were found to have antibodies for *Lept. pomona*. Peterson[11] in Australia demonstrated leptospiras in both cattle and pigs from one farm. In this work leptospirosis was shown to have transferred readily from infected pigs to susceptible ones and from infected pigs to calves. The ease of this transmission is understandable when consideration is given to the tremendous number of organisms eliminated by carrier pigs. Pigs exposed to infected calves, however, did not contract leptospirosis. Failure to achieve transmission from infected calves was probably due to the relatively small numbers present in their urines. This is not to imply that under certain conditions pigs could not become infected from calves. It has been amply demonstrated that infection can be transmitted between calves.[16] In areas where swine and cattle cohabit the same premises, therefore, it seems logical, in outbreaks of bovine leptospirosis, to incriminate swine whenever possible as the source of infection until proven otherwise. It should be borne in mind, of course, that in areas where cattle are kept separated, the disease is entirely capable of spreading through a herd without the mediation of swine.[17]

This brings up the important question of the natural host for *Lept. pomona*. Although rodents are the hosts for most species of *Leptospira*, the evidence reported to date has not indicated that this is so for *Lept. pomona*.[8] Infection by this organism has been reported in other animals, including the

17. Little, R. B. and Baker, J. A. 1950, J.A.V.M.A. **116**: 105–111.

dog,[6] rat,[8] and horse;[18] however, most information seems to point to either swine or cattle as the probable host for this organism. The infection in cattle, although important in the United States and Australia, does not appear to be as widespread on a world-wide basis as it does in swine. If the growth and multiplication of the leptospiras in the host are criteria, the organism is better adapted to pigs, as evidenced by the large numbers excreted in the urine as well as by the length of the carrier states. Cattle have not been shown to harbor leptospiras for longer than 3 months.[16,19] Thus, it appears that swine may be the natural hosts for *Lept. pomona*.

SUMMARY

Leptospira pomona produced infection in all of 25 pigs inoculated subcutaneously and in all of 7 pigs inoculated intranasally, but failed to infect 6 of 7 pigs when administered orally. Except for 2 pigs that developed severe illness and jaundice, all infected animals appeared normal, showing only a slight elevation in temperature. Following inoculation, leptospiras were found in the blood only in the early stage of infection and in the urine in the later stages. Their persistence in the urine correlated with the finding of lesions in the kidneys. Infected pigs excreted numerous leptospiras in their urine over long periods of time, which is in contrast to calves that shed relatively few organisms over a shorter period of time. Transmission of infection by contact exposure occurred readily from infected pigs to susceptible ones and to calves, but not from calves to pigs. Infection, therefore, should be widespread in the swine population, and 22% of 285 swine that came to market were found to have antibodies for *Lept. pomona*. These facts emphasize the importance of swine as reservoirs for *Lept. pomona*.

18. Roberts, S. J., York, C. J. and Robinson, J. W. 1952, J.A.V.M.A. **121**: 237–242.
19. Sutherland, A. K. and Simmons, G. C. 1949, Australian Vet. J. **25**: 197–202.

FURTHER KLEBSIELLA CAPSULE TYPES

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Julianelle (1926), working with Friedlander's bacillus, demonstrated by agglutination, agglutinin-absorption, mouse-protection and precipitation tests, three sharply defined capsular types A, B, and C, and a heterogeneous group, X. These types were represented by 30 strains, most of which were associated with lobar pneumonia in human subjects.

Edwards, in 1929, showed that 5 cultures of *Bacterium aerogenes* isolated from soil, water and milk were culturally, biochemically and serologically identical with type B Friedlander's bacillus as described by Julianelle. He concluded that *Bact. aerogenes* is so closely related to Friedlander's bacillus that they should be classified in the same genus.

In 1936, the number of types of this group was increased by the work of Goslings and Snijders to 6, the 3 new ones (D, E, and F) being isolated from cases of ozaena. Kauffmann (1949), working with 130 cultures of respiratory, urinary and other sources, increased the known number of capsular (K) antigenic types of the klebsiella group by 8 to a total of 14. All of these new types, except type 7, were represented by cultures freshly isolated from urine.

In their 1952 paper, Edwards and Fife described types 1 to 57 of the klebsiella group, including Julianelle's (1926) original types (A, B and C, now 1, 2 and 3), Goslings and Snijder's (1936) types (4 to 6), Kauffmann's (1949) types (7 to 14), Worfel and Ferguson's (1951) type (15), Brooke's (1951) types (26 to 49) and Edwards and Fife's new types, which became types 16 to 25 and 50 to 57. Three further strains were supplied by Edwards to the present author since this last paper was published, which, together with three additional types recently recognized by Edwards and Kauffmann (Edwards, personal communication), make a total of types 1 to 63 so far recognized.

In view of the difficulty encountered by Kauffmann (1949) in the determination of O antigens of the klebsiella group and since, in addition, overlapping reactions occur between klebsiella K and O antigens and those, for example, of *Escherichia coli* (Kauffmann, 1949), it seems impracticable to base a classification of klebsiella strains on serology alone. It is therefore necessary to take into account the cultural and biochemical characters of this group. That these are not always consistent with serological findings was pointed out by Vahlne (1945), who cited two serologically identical strains, one of which culturally corresponded to *Esch coli*, and the other to *Bacterium lactis aerogenes*, while one of Escherich's type strains of *Bact. lactis aerogenes* proved to belong to O

Received for publication July 1, 1953.

Acknowledgments are due to Professor T. J. Mackie and Dr. J. P. Duguid of this Department, and to Dr. P. R. Edwards of the Communicable Disease Center, Atlanta, Georgia, for their advice and criticism. Dr. Edwards was kind enough to ascertain that the proposed new types were serologically distinct from the recently recognized types 60 to 63.

In referring directly to previous original articles, the nomenclature used by their authors has been adhered to.

group 9 in the antigenic schema for colon bacilli.

This overlapping does not present any insuperable difficulties in the biochemical and morphological differentiation between the klebsiella-aerogenes group and the genus *Escherichia* (Kauffmann, 1949). On the other hand, biochemical differentiation between individual members of Kauffmann's proposed new klebsiella genus is on a more doubtful basis, and such differences as there are show such variability that no sufficient reason exists for ascribing generic status to such differing members Thus, in support of the proposals of Edwards (1929) and Kauffmann (1949) for this new genus, one may cite the recent findings of Renoux and Terdjman (1952), who were unable to demonstrate, by virulence for mice as well as by morphological, cultural and biochemical characters, any criteria which could be used to distinguish *Aerobacter aerogenes* from *Klebsiella pneumoniae*. Dr. Cowan, Curator of the National Collection of Type Cultures (NCTC) in London, in a personal communication, also lends his support to the idea that all cultures labelled *Bact. aerogenes* should fall into the genus *Klebsiella*. Although Henriksen (1952) has suggested that there might be scope for biochemically defined subgroups of the genus *Klebsiella*, particularly in the case of rhinoscleroma group, he has not challenged the proposed *Klebsiella* genus as such.

MATERIALS AND METHODS

Coliform strains were obtained from the following sources: (a) Urinary infections (9 strains of *Klebsiella*). (b) Soil and water, isolated from milk (9 strains of *Klebsiella*). (c) Collection of Dr. J. P. Duguid (Bacteriology Department, Edinburgh University), from feces, infected urine, wounds and milk, whose individual sources are not known (14 strains of *Klebsiella*). (d) Julianelle's types A, B and C of Friedlander's bacillus, as represented by NCTC numbers 5054, 5055 and 5056. (e) Wilson's (1935) biochemical types I and II of *Bact. aerogenes*, represented by NCTC numbers 8172 (type I), and 5939 and 8167 (type II); NCTC-5939 was supplied by the National Collection in London as *Bact. aerogenes* type II, but the culture was discarded by the Curator in 1950. (f) Three strains of *Aerobacter cloacae*, represented by NCTC5936, A217 and U8. A217 comes from Dr. Duguid's collection and U8 is a urinary strain. NCTC5936 was supplied as such by the National Collection in 1949 and will be described under that number throughout this paper, but Dr. Cowan has since changed its number to 8348. This strain is classed by him as *Bact. aerogenes* because of its ability to hydrolyse urea and its failure to liquefy gelatin. However, since *A. cloacae* sometimes loses its power to liquefy gelatin (Bergey's Manual, 1948) and since NCTC-5936 ferments glycerol with acid and no gas and is a motile noncapsulate organism, the author feels justified in classing it as *A. cloacae*. This also accords with the serological findings, since its slime hapten appears to be the same as that of A217 and U8, both of which are undoubted *A. cloacae* as defined in Bergey's Manual (1948). (g) Capsulate *Esch. coli* from urine (2 strains). (h) Capsule types of klebsiella 1 to 60 supplied by Edwards.

Biochemical tests.—The tests indicated in table 1 were carried out by the techniques described by Wilson (1935).

Preparation of K antigen.—The surface of a lactose agar plate was evenly inoculated with a loopful of growth from an agar slope. The medium contained 0.5% lactose and 0.5% peptone, giving a sugar-peptone ratio of 1:1, which is sufficient to give good but not excessive growth of capsules (Duguid, 1948). After incubation for 18 to 24 hours at 37 C, the resulting growth was emulsified in saline to give Brown's opacity standard 3 for immunizing purposes (i.e., approximately 10^9 organisms per ml) and 1 for reagent in agglutination and capsule-swelling tests (i.e., 4×10^8 per ml). After checking for capsules by moist India ink preparations, the latter suspension was preserved by the addition of 0.08% sodium azide.

Preparation of capsular antiserums.—Large healthy rabbits were used. One-tenth ml of unpreserved suspension was injected intravenously, and subsequent doses of 0.2, 0.4, 0.6 ml etc. were given at intervals of 4 to 5 days. Before each initial injection and at suitable intervals thereafter, the rabbits were bled and the serum examined for capsule-swelling antibodies. The period required to obtain a good antiserum varied from 3 to 6 weeks. The rabbits were bled from an ear vein a week after the final injection. Serums

TABLE 1.—*Biochemical and morphological characters and sources.*

Strain	Motility	Eijkman	Voges-Proskauer	Methyl red	Glucose	Lactose	Dulcitol	Saccharose	Mannite	Inositol	Glycerol	Citrate utilization	Indole	Gelatin liquefaction	Capsule
Source: NCTC															
NCTC 5939	−	−	+	−	+	+	+	+	+	+	+	+	+	−	+
NCTC 5936	+	−	+	−	+	+	−	+	+	⊥	⊥	+	−	−	−
NCTC 5054	−	−	±	+	⊥	⊥	−	⊥	⊥	⊥	⊥	+	−	−	+
NCTC 5055	−	−	+	+	+	+	−	+	+	+	+	+	−	−	+
NCTC 5056	−	−	−	+	+	+	+	+	+	+	+	+	−	−	+
NCTC 8167	−	−	+	−	+	+	−	+	+	+	+	+	+	−	+
NCTC 8172	−	+*	+	−	+	+	−	+	+	+	+	+	−	−	+
Source: Collection of Dr. J. P. Duguid A no. 1, 3, 4, 8, 10, 14, 17, 20, 22, 23, 25, 26, 27, 29	−	−	+	−	+	+	−	+	+	+	+	+	−	−	+
A217	+	−	+	−	+	+	−	+	+	−	⊥	+	−	+	−
Source: Urinary infection															
U3	+	+	−	+	+	+	−	−	+	−	+	−	+	−	+
U8	+	−	+	−	+	+	−	+	+	−	⊥	+	−	+	+
417	−	+	−	+	+	+	−	−	+	−	+	−	−	−	+
U4, 499, 625, 411, 291, 494, 495, 930, 498	−	+	+	−	+	+	−	+	+	+	+	+	−	−	+
Source: Milk															
SW4	−	−	−	+	+	⊥	−	+	+	⊥	+	+	−	−	+
SW5	−	−	+	−	+	+	−	+	+	⊥	⊥	+	−	−	+
903	−	−	−	+	+	+	+	+	+	+	+	+	−	−	+
434.1	−	−	+	−	+	+	−	+	+	−	⊥	+	−	−	+
438.3a	−	−	+	−	+	+	−	+	+	+	+	+	−	−	+
265.1	−	−	+	−	+	+	+	+	+	+	−	+	−	−	+
326.1, 264.1, 889	−	−	+	−	+	+	−	+	+	⊥	+	+	−	−	+

All strains gave mucoid growths, those not capsulate producing loose slime instead.
⊥, Acid only.
+, Acid and gas in fermentation reactions.
±, Doubtful reaction.
* Negative at 45 to 46 C.

were preserved by the addition of 0.08% sodium azide and were stored at 4 C.

Capsule-swelling reactions.—Serial dilutions of serum in saline, 1:2, 1:4 etc., were made, and a drop of each dilution mixed on a slide with a drop of antigen. A coverslip was placed in position and carefully sealed to the slide with melted vaseline. Examination was then carried out under the oil-immersion lens. Titers given refer to initial dilutions, before mixing with antigen. If the capsule-swelling titers of any antiserum to two organisms differed by a factor of 16 or more, they were considered to be serologically distinct from one another, without the necessity for absorption tests. If the difference in titers was less than 16-fold, absorption tests were carried out. If capsular titers of the two organisms were comparable, however, the two organisms were sometimes placed in the same serological type even if absorption tests proved them not to be absolutely identical (Edwards, personal communication).

Tube agglutination reactions.—Doubling dilutions of serum from 1:5 upwards were made in normal saline, equal volumes of antigen added and the mixtures transferred to agglutination tubes, as in the Widal reaction. The tests were incubated for 2 hours at 37 C, read, and placed in the refrigerator overnight at 4 C. Final readings were then made. Titers refer to final dilutions of serum, after addition of antigen.

Absorption tests.—A dense suspension of the absorbing organism was prepared by emulsifying in 1 to 3 ml saline the growth from a lactose agar plate which had been incubated for 24 hours at 37 C. To 0.8 ml undiluted serum, 0.4 to 0.8 ml of suspension was added drop by drop, while shaking the mixture vigorously. A rough guide as to the amount of suspension required was given by the cessation of immediate macroscopic agglutination as excess suspension was added. The mixture was incubated for half an hour in the 37 C water bath and then centrifuged. The supernatant was checked for absence of capsule swelling of the absorbing strain, and if capsule swelling still took place, the process was repeated until absorption was complete.

RESULTS

Biochemical and morphological characters.—These are given in table 1. It will be seen that certain deviations occur from the typical klebsiella-aerogenes organism as represented by previous authors (Wilson, 1935; Vahlne, 1945; Kauffmann, 1949; Edwards and Fife, 1952). Apart from the *A. cloacae* and escherichia strains, 12 strains differed from the typical in respect of 1, 2, or 3 characters. All the organisms of the series utilize citrate, except for the escherichia strains, and all are capsulate except 2 of the 3 *A. cloacae* strains (NCTC-5936 and A217). U8 is interesting in that its characters include both motility and the ability to produce capsules.

Serological reactions.—Capsule swelling and agglutination test results are given in tables 2 and 3 for 6 new klebsiella serums versus Edwards' K types 1 to 60 and the 6 new types which emerged from the present series. Other tests not included in these tables were also carried out, namely capsule-swelling and agglutination reactions between a further 12 klebsiella serums, 2 escherichia and 1 *A. cloacae* serum versus K types 1 to 60, the 6 new types of klebsiella, the 2 escherichia strains and the *A. cloacae* strain. In interpretation of these results, particular attention was paid to the capsule-swelling reactions, whose titers were less affected than the agglutination titers by the differing amounts of capsular substance produced by individual strains. Also, in some of the agglutination tests there was suspicion that O agglutination was occurring in addition to the K reaction, thus prolonging the end point towards the higher titers characteristic of the former type of reaction. Thus the 16-fold difference in capsule-swelling titers of a pair of strains, which was taken to indicate a definite difference in type, did not always occur in the corresponding agglutination titers.

TABLE 2.—*Capsule-swelling reactions.**

Antigens	Type numbers of antiserums of new klebsiella strains					
	64	65	66	67	68	69
1	—	—	—	—	—	—
2	—	—	—	—	—	—
3	—	—	—	—	16	—
4	—	—	—	—	—	—
5	—	—	—	—	—	—
6	—	—	—	—	—	—
7	—	8	—	—	—	—
8	—	—	—	—	—	—
9	—	—	—	—	—	—
10	—	—	—	—	—	—
11	—	—	—	—	—	—
12	4	—	—	—	—	—
13	4	—	—	—	—	—
14	32	—	—	—	—	—
15	—	—	—	—	—	—
16	—	—	—	—	—	—
17	—	—	—	—	—	—
18	—	—	—	—	—	—
19	—	—	—	—	—	—
20	—	—	—	—	—	—
21	4	—	—	—	4	16
22	—	—	—	—	—	—
23	—	—	—	—	—	—
24	—	—	—	—	—	—
25	—	—	—	—	—	—
26	—	—	—	—	—	—
27	—	—	—	—	—	—
28	—	—	—	—	—	—
29	—	—	—	—	8	—
30	—	—	—	—	—	32
31	4	—	—	—	8	—
32	—	—	—	—	—	—
33	4	—	—	—	4	8
34	—	—	—	—	—	—
35	—	—	—	—	—	—
36	8	—	—	—	4	—
37	—	—	—	—	—	—
38	—	—	—	—	—	—
39	—	—	—	—	—	—
40	—	—	—	—	—	—
41	—	—	—	—	—	—
42	—	—	—	—	—	—
43	—	—	—	—	—	—
44	—	—	—	—	—	—
45	—	—	—	—	—	—
46	—	—	—	—	—	—
47	—	—	—	8	—	—
48	—	—	—	—	—	—
49	—	—	—	—	—	—
50	—	—	—	—	—	—
51	—	—	—	—	—	—
52	—	—	—	—	—	—
53	—	—	—	—	—	—
54	—	—	—	—	—	—
55	—	—	—	—	—	8
56	—	—	—	—	—	—
57	—	—	—	—	—	—
58	—	—	—	—	—	—
59	—	—	—	—	—	—
60	—	—	—	—	—	—
64	64	—	—	—	4	—
65	—	128	—	—	—	—
66	—	—	16	—	—	—
67	—	—	—	256	—	—
68	—	—	—	—	64	—
69	—	—	—	—	—	128

—, Negative.
* Titers are given as the reciprocal of final dilutions.

In the case of strains giving significant cross reactions in capsule-swelling reactions, absorption tests were carried out to determine the types to which these strains should be allocated. The following pairs of strains were thus

proved to have identical capsular antigens: A1 and A3; A3 and K54; A22 and U4; NCTC8167 and K18; NCTC5939 and K36; 326.1 and K42. Other strains not proved to be absolutely identical were nevertheless sufficiently similar to be allocated to types as follows: A4 to type 8; A22, 499, 625, 495, 930, 411, 291, 494 and 498 to type 30; 903 to type 39; 434.1 to type 41; A8, A10, A14, A17, A20, A23, A25, A26 and A27 to type 54; A29 to type 57; SW5 to type 65; NCTC5936 and A217 to *A. cloacae* type. Strains shown by absorption to be serologically distinct included: NCTC5054 (K1) and K54; NCTC5055 (K2) and 889 (K69); NCTC5056 (K3) and 265.1 (K68); A4 (K8) and K27; A22 (K30) and K13; A22 and 889 (K69); NCTC 5939 (K36) and K14; 903 (K39) and K10; 434.1 (K41) and K50; 326.1 (K42) and K12; 326.1 and K29; A3 (K54) and 417; NCTC8172 (K64) and K36; NCTC8172 and K14; 265.1 (K68) and K29; 265.1 and K31; 889 (K69) and K21; U8 and K21.

From tables 2 and 3, together with the absorptions given above, 6 new types emerged, which it is proposed to designate as numbers 64 to 69, inclusive. The following are the original numbers of the new klebsiella strains together with their proposed K numbers: NCTC8172 (K64); SW4 (K65); 438.3a (K66); 264.1 (K67); 265.1 (K68); 889 (K69). All these strains except NCTC8172 were isolated from milk.

Of the 35 hitherto untyped klebsiella strains of the series, 7 fell into the 6 new types (64 to 69) and 28 were fitted into types 1 to 63. The latter represented 9 different K types, viz., numbers 8, 18, 30, 36, 39, 41, 42, 54 and 57. Of these 28 strains, 2, representing K types 18 and 36, respectively, were National Collection cultures, so that only 7 previously described K types were represented by the Edinburgh klebsiella strains isolated in this laboratory. These 7 types encompassed 26 of the 32 Edinburgh strains.

NCTC numbers 5054, 5055, 5056 were confirmed as being the same as types 1, 2 and 3. As indicated above, NCTC numbers 8167 and 5939 were proved identical with K types 18 and 36, respectively. One National Collection cul-

TABLE 3.—*Agglutination reactions.*

Antigens	Type numbers of antiserums to new klebsiella strains					
	64	65	66	67	68	69
1	10	—	—	—	—	—
2	—	—	—	—	—	40
3	—	—	—	—	40	—
4	—	—	—	—	—	—
5	—	—	—	—	—	—
6	—	—	—	—	20	—
7	—	20	—	—	—	—
8	—	—	—	—	—	40
9	—	—	—	—	—	—
10	—	—	—	—	—	—
11	—	—	—	—	10	20
12	10	—	—	—	10	—
13	10	—	—	—	20	—
14	160	—	—	—	—	—
15	—	—	—	—	—	20
16	—	—	—	—	—	—
17	10	—	—	20	—	—
18	—	—	—	—	—	—
19	—	—	—	—	—	—
20	—	—	—	—	—	—
21	20	—	—	—	40	20
22	—	—	—	—	—	—
23	—	—	—	—	—	20
24	—	—	—	—	—	—
25	—	—	—	—	—	—
26	—	—	—	—	10	—
27	—	—	—	—	10	—
28	—	—	—	—	—	—
29	40	—	—	—	80	20
30	—	—	—	—	10	40
31	40	—	—	—	40	—
32	—	—	—	—	—	—
33	10	—	—	—	20	20
34	—	—	—	20	—	—
35	—	—	—	—	10	—
36	40	—	—	—	40	—
37	—	—	—	—	—	—
38	—	—	—	—	—	—
39	—	—	—	—	—	—
40	—	—	—	—	—	—
41	—	—	—	—	—	—
42	10	20	—	—	40	—
43	20	—	—	—	—	—
44	—	—	—	—	—	—
45	—	—	—	—	—	—
46	—	—	—	—	—	—
47	20	—	—	80	—	—
48	—	—	—	20	—	—
49	—	—	—	—	—	—
50	—	—	—	—	—	—
51	—	—	—	—	—	—
52	—	—	—	—	—	—
53	—	—	—	—	—	—
54	—	—	—	—	—	—
55	—	—	—	—	—	—
56	20	—	—	—	20	—
57	—	—	—	—	—	—
58	—	—	—	—	—	—
59	—	—	—	—	—	20
60	—	—	—	—	—	—
64	160	—	—	—	—	—
65	—	320	—	—	—	—
66	—	—	40	—	—	—
67	—	—	—	320	—	—
68	10	—	—	—	320	—
69	—	—	—	—	10	320

See footnotes of table 2.

ture belonged to a new klebsiella type (8172 was type 64).

Of the Edinburgh klebsiella strains, 10 out of 32 (31%) were type 30, 11 (34%) were type 54, and 2 were type 65. The remaining types were represented only by single strains. Two K types therefore encompassed 21 out of 32, or 66% of the total number of locally isolated strains. Neither of these two main serological groups included any of the NCTC strains examined.

DISCUSSION

The problem of the definition of membership of the genus *Klebsiella* as against *Escherichia* is a difficult one. The impracticability of distinguishing the genera serologically, particularly in determining klebsiella O antigens, emphasizes the need for more general agreement as to the biochemical and morphological characters defining them.

The conception of Wilson (1935), who ignored inositol fermentation, of a differentiation based on methyl red, Voges-Proskauer, citrate utilization and Eijkman reactions has by no means been adhered to by later workers, and emphasis has been displaced from this combination of reactions to the results of inositol and adonitol fermentation, citrate utilization and indole production tests. Thus Vahlne (1945) laid greatest stress on citrate utilization as a criterion for membership of the genus *Klebsiella*, whereas Kauffmann (1949) wrote that Gram-negative nonmotile mucoid bacilli which ferment inositol and adonitol but do not produce indole were "most likely to be *Klebsiella*," while Edwards and Fife (1952) gave the chief *Klebsiella* criteria as: methyl red, hydrogen sulphide and indole tests negative; dulcitol fermentation negative; Voges-Proskauer, citrate, urea, tartrate and nitrite tests positive; with fermentation of various sugars including inositol, adonitol and salicin. Of the 256 cultures examined by Edwards and Fife, however, 151 differed from the above pattern in one or more respects, including 31 cultures which were methyl red positive, 25 which were Voges-Proskauer negative, etc.

In the present work, a combination of citrate utilization and ability to ferment inositol, along with negative indole and Eijkman reactions, was taken as allowing the inclusion of a nonmotile strain in the klebsiella group, even if Voges-Proskauer was negative and methyl red positive. Thus, strains SW4, NCTC5056, and 903 were included, although these were all Voges-Proskauer negative and methyl red positive. U3 and 417, on the other hand, were inositol and citrate negative, and Eijkman positive as well, and they were therefore taken as *Escherichia*.

Although Kauffmann laid some stress on the indole reaction as a criterion, Wilson's type II *Bact. aerogenes* (1935), as exemplified by NCTC5939, while indole and dulcitol positive, is nevertheless citrate and inositol positive, Voges-Proskauer positive and methyl red negative. It therefore seems that such indole positive strains should be included in a klebsiella series since the majority of important criteria are satisfied. On the other hand, as Kauffmann (1949) in his summary of criteria gives lack of motility as a requirement for klebsiellas, it was decided to exclude NCTC5936, A217 and U8, which are motile, mucoid organisms, although they are typical as regards methyl red, Voges-Proskauer, citrate utilization, indole and Eijkman reactions.

The serological results are in keeping with the impression given by the work of Edwards and Fife (1952), namely that the predominating capsular types vary considerably from one country to another. These workers found that the

8 types established by Kauffmann in Denmark (types 7 to 14) were represented by only 35 out of 256 cultures isolated in the United States, while the marked predominance of types 8, 9 and 10 noted by Brooke and Kauffmann was not evident in the American study. Thus, Kauffmann (1949) assigned 93% of urinary strains to these 3 types, while Brooke (1951) included 63% of his urinary strains in them. Edwards and Fife (1952), on the other hand, found only 16% of urinary cultures to be included in these types, while in the present series none out of 10 urinary strains could be fitted into these types.

It is of interest to note that the two escherichia cultures also studied both gave overlapping of K antigens with various klebsiella types. In the case of 417, particularly, which is biochemically *Esch. coli* type II (Wilson, 1935), it could only be distinguished definitely from A3 (K type 54) by cross-absorption tests. These results compare with those of Kauffmann (1949), who found that klebsiella type 10 was almost identical with escherichia type 39, while klebsiella types 7, 8 and 11 had partial K antigens in common with escherichia types 55, 34 and 37, respectively.

SUMMARY

Forty-three mucoid strains of coliform bacilli from various sources were divided biochemically into 38 klebsiella strains, 2 escherichia strains and 3 *Aerobacter cloacae* strains.

Of 35 hitherto untyped klebsiella strains, 6 new types were distinguished by capsule-swelling, agglutination and absorption tests, from previously known types 1 to 63.

Two main serological groups emerged, namely type 54, which included 34% of the Edinburgh strains, and type 30, which included 31%. This contrasts with the quite different distributions of types which have been found in Denmark and the United States.

Biochemical and morphological criteria for the klebsiella group are discussed.

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AGGLUTINATION OF BACTERIA (*HEMOPHILUS PERTUSSIS* AND *SALMONELLA TYPHOSA*) IN VITRO BY HUMAN BLOOD LYMPHOCYTES AND CYTOPLASMIC EXTRUSIONS OF LYMPHOCYTES

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Direct morphological relationships between bacteria and lymphocytes have been obscure for a long time, for the phenomenon of phagocytosis of and taxis toward foreign bodies by lymphocytes has not been confirmed under the microscope. To our knowledge, the only study which has clearly proved such a relationship is that of Hayes, Dougherty and Gebhardt (1951). These authors demonstrated the agglutination of *Salmonella typhosa*, *Salmonella typhimurium* and *Pseudomonas aeruginosa* by lymphocytes in air-dried tissues which had been obtained from mice immunized by the same antigens and spread on glass slides. The photomicrographs in their paper undoubtedly represent this phenomenon, but it seems somewhat uncertain whether the cells demonstrated are all lymphogenous. Reis, Mertens, and Ehrich (1950) reported a phenomenon, closely resembling that of Hayes et al, of lymphoid cells which had been taken out of the popliteal lymph nodes of immunized rabbits and which were believed to belong to the plasma cell series.

In unstained or supravitally stained specimens, it is impossible to observe the delicate structures of cytoplasm and nucleus and quite difficult to differentiate morphologically similar cells. The leukocytes of the blood without marked pathological changes are better materials to identify types of cells than are cells derived from the skin, lymph nodes, bone marrow, etc. Human blood specimens which are spread on glass slides in thin films, fixed by the dry-heat method and stained with proper solutions give the best opportunity to observe fine details of both blood cells and bacteria. This paper describes the morphological relationships in vitro between the bacteria (*Hemophilus pertussis* and *S. typhosa*) and the blood lymphocytes of patients with whooping cough and typhoid fever, respectively, and of normal human beings.

Received for publication July 1, 1953.

MATERIALS AND METHODS

Clinical material.—Two patients with whooping cough were studied: a 4-year-old girl and a 2-year-old boy in the same family. The boy was infected by his sister and developed the illness about 10 days later. The experiment was performed at the same time on both children about 30 days after the onset of her illness.

One patient with typhoid fever was also studied. This was a 3-year-old boy in the convalescent stage, from whom specimens were obtained about a month after the onset of the illness. The Widal test was positive to a titer of 800.

Control subjects included 2 healthy infants, a 13-month-old boy and an 11-month-old girl, neither of whom had been infected with whooping cough since their birth, nor immunized against this disease, and 2 healthy adults, a 39-year-old man and a 25-year-old woman. The latter had had whooping cough in their early childhood but had no history of typhoid for at least 4 years.

The agglutination test against *H. pertussis* and the Widal test of the controls were all negative.

Bacterial suspensions.—These contained about 60 billion living bacteria (*H. pertussis or S. typhosa*) per cc of serum or of physiological saline. The suspensions were homogenized in a sterile agate mortar and observed microspically prior to experiment. *H. pertussis* had been cultured on Bordet-Gengou's medium for 48 to 72 hours and

S. typhosa on agar nutrient medium for 24 to 48 hours.

Methods.—One to five cc of venous blood from the ill or healthy subjects were poured into a small test tube and defibrinated with a small glass rod or treated with heparin (approximately 0.007%). The fresh bacterial suspensions were dropped into the blood with a small syringe to one-twentieth the volume of blood. The blood was stirred thoroughly with a glass rod and placed in an incubator at 37 C. All procedures were done aseptically.

The test tubes were removed from the incubator at varying times. After stirring again thoroughly with a glass rod, a drop of the blood was put on a glass slide and spread to a thin smear. These smears were dried in air and placed in a high-temperature box to fix by dry-heat (160 C, for 5 minutes). Fixations with methyl alcohol and other liquid fixatives were also tried but did not give good results in revealing the fine surface structures of lymphocytes.

The preparations were usually stained with Field's solution in order to avoid influence of alcohol and to shorten staining time.

The blood smear was placed on a mechanical stage of a microscope, and the number of all the lymphocytes appearing in the several hundred microscopic fields and of the bacteria gathered around them was determined. In the same specimen all erythrocytes and bacteria observed in microscopic fields narrowed by inserting an iris in the ocular lens, were counted (table 1).

RESULTS

When a suspension of *H. pertussis* was added to the blood of patients suffering from whooping cough, the bacteria markedly gathered themselves around the lymphocytes. The phenomenon agglutination of bacteria by lymphocytes was already quite distinctly observed 20 minutes after the addition of the bacteria and reached a peak in about 90 minutes. Twenty hours later many of the bacteria had degenerated and their number had decreased markedly. In both children the results were very similar.

TABLE 1.—*Relationship between* H. pertussis *and the lymphocytes of children suffering from whooping cough.*

Case	Time	Average number of bacteria agglutinated per lymphocyte	Average number of bacteria per erythrocyte
I	20 min.	7.3	0.49
	45 min.	8.6	0.33
	1.5 hr.	15.1	0.33
	3 hr.	11.6	0.34
	20 hr.	2.5	0.20
II	20 min.	7.8	0.33
	45 min.	9.5	0.33
	1.5 hr.	14.6	0.33
	3 hr.	11.8	0.36
	20 hr.	1.8	0.15

From 170 to 264 lymphocytes and from 1500 to 2800 erythrocytes were observed at each interval to obtain the above ratios.

TABLE 2.—*Relationship between* H. pertussis *and the lymphocytes, in vitro, of healthy adults who have had whooping cough in their early childhood.*

Case	Time	Average number of bacteria agglutinated per lymphocyte	Average number of bacteria per erythrocyte
I	20 min.	1.5	0.26
	1.5 hr.	2.7	0.24
	6 hr.	1.1	0.26
II	10 min.	1.4	0.33
	30 min.	1.8	
	1 hr.	2.3	
	2 hr.	1.4	0.22
	4 hr.	1.0	

Number of lymphocytes observed: 301 to 626.
Number of erythrocytes observed: 3500 to 5600.

The agglutination of *H. pertussis* by the lymphocytes of normal blood was weaker than that of the patients; the phenomenon was evident in this case as well (table 2 and 3). Normal lymphocytes, even though they may carry no specific antibodies against *H. pertussis*, must have an affinity for the bacteria.

When the suspension of *S. typhosa* was added to the blood obtained from a patient with typhoid fever, the phe-

TABLE 3.—*Relationship between* H. pertussis *and the lymphocytes in vitro, of children, who have had no history of whooping cough.*

Case	Time	Average number of bacteria agglutinated per lymphocyte	Average number of bacteria per erythrocyte
I	30 min.	3.06	0.46
	1 hr.	3.72	0.54
	2 hr.	2.69	0.41
II	30 min.	2.37	0.62
	1 hr.	4.51	0.59
	2 hr.	1.67	0.42

Number of lymphocytes observed: 147 to 263.
Number of erythrocytes observed: 1120 to 2480.

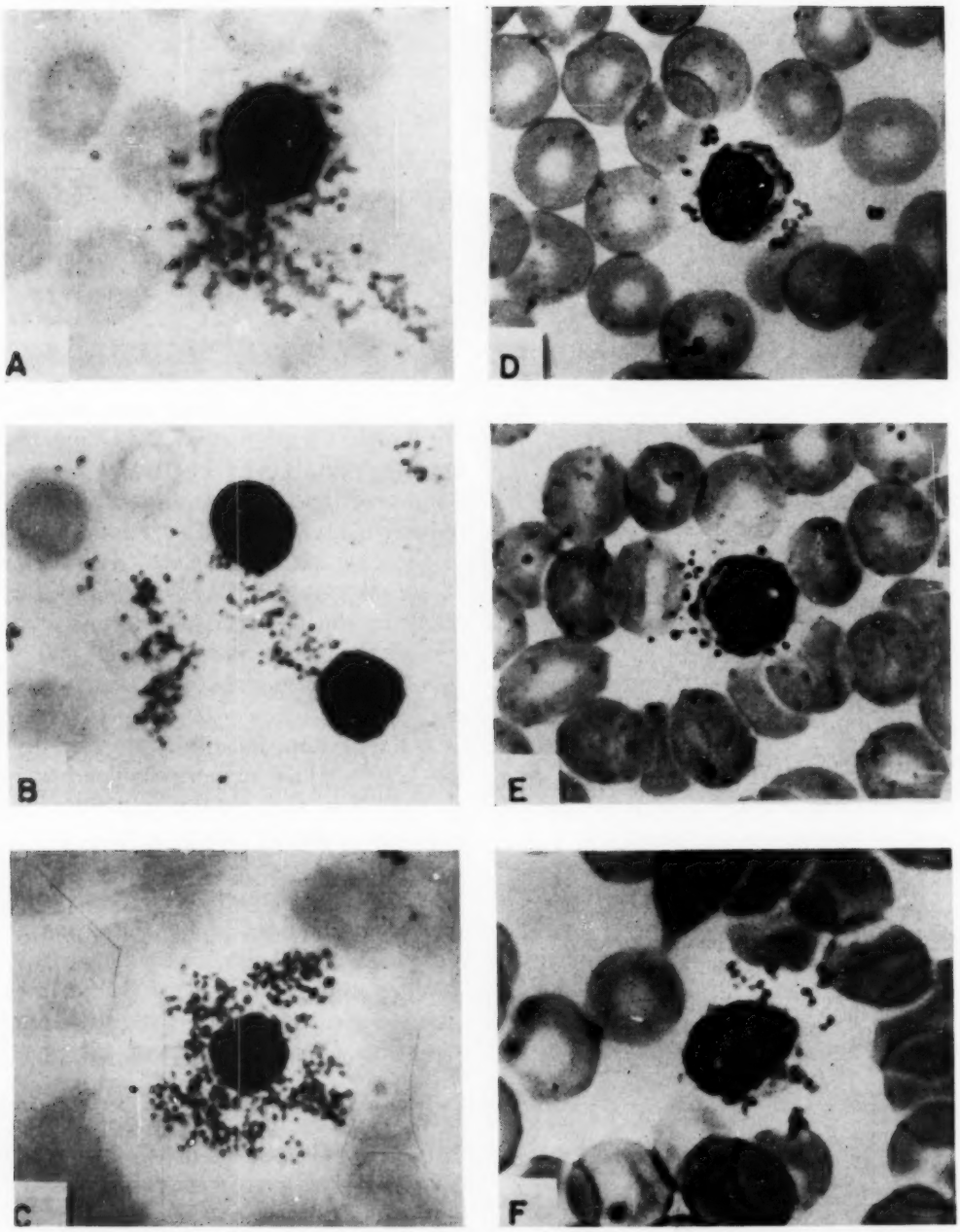

FIG. 1.—A, B, C. Lymphocytes from blood of children suffering from whooping cough to which a suspension of *H. pertussis* was added in vitro, with smears made at intervals thereafter.

D, E, F. Similar preparations from blood of normal children.

TABLE 4.—*Relationship between* S. typhosa *and the lymphocytes in convalescence of typhoid fever.*

Time	Average number of bacteria agglutinated per lymphocyte	Average number of bacteria per erythrocyte
5 min.	0.7	0.41
3 hr.	1.8	0.07
18 hr.	0.4	0.01

Number of lymphocytes observed: 155 to 290.
Number of erythrocytes observed: 2680 to 3840.

nomenon was likewise confirmed, although it was not so noticeable as in the cases of whooping cough; while the lymphocytes of the healthy control scarcely agglutinated *S. typhosa*, which were found evenly distributed in the specimens (table 4 and 5). In the last case the number of bacteria per lymphocyte was much less than that per erythrocyte, 5 and 30 minutes after the addition of bacteria. These apparently peculiar results are due to the fact that all bacteria in the microscopic fields were counted whether they were in contact with erythrocytes or not.

All lymphocytes in the same material did not show the same affinity for bacteria. There were always fairly large numbers of lymphocytes which showed no contiguity to bacteria in the blood. The average percentage of these lymphocytes against *H. pertussis* were 34.6% (21.6% to 55.3%) in the patients with whooping cough and 51.4% (27.4% to 78.8%) in the healthy children and adults, while that against *S. typhosa* were 90.7% (88.3% to 94.2%) in the patient with typhoid fever and 96.6% (92.7% to 98.7%) in the healthy control. Whether antibodies were present or not, the number of lymphocytes agglutinating *H. pertussis* bacteria was relatively large, while only a few lymphocytes agglutinated *S. typhosa*.

TABLE 5.—*Relationship between* S. typhosa *and normal lymphocytes.*

Time	Average number of bacteria agglutinated per lymphocyte	Average number of bacteria per erythrocyte
5 min.	0.09	0.37
30 min.	0.08	0.33
3 hr.	0.21	0.10
18 hr.	0.04	0.09

Number of lymphocytes observed: 145 to 230.
Number of erythrocytes observed: 4000 to 4500.

The combination of agglutinated bacteria and lymphocytes was apparently quite strong in that it was not much distributed by the procedures of stirring, agitating and making smears of the blood (fig. 1). The bacteria agglutinated by lymphocytes degenerated and disappeared more rapidly than those ingested by other leukocytes.

Because the lymphocytes were more resistant than the neutrophiles to bacteria, the blood pictures of the specimens gradually changed after the addition of bacteria. The percentage of lymphocytes gradually increased, although the absolute number decreased slightly.

In the preparations which were adequately fixed by means of the dry-heat method, the lymphocytes could be observed agglutinating the bacteria by fine cytoplasmic extrusions radiated from the cell surface. These extrusions were cilia-like or membranous in shape and stained generally light rosy pink with Field's solution or other eosin-azure-methylene-blue solutions, in contrast with the blue cytoplasm of lymphocytes. The free ends of the extrusions were so fine that they often disappeared in the surrounding plasma. The cilia-like extrusions occasionally reached over 10 μ in length. The lymphocytes in simple smears without addition of bacteria were also often observed radiating similar extrusions when the same procedures of fixation and staining were done. Such features of the cell surface were especially prominent in lymphocytes and were very rarely seen in other leukocytes and in erythrocytes of normal blood.

DISCUSSION

In discussing the relationships between human blood cells and bacteria, it should be pointed out that normal lymphocytes have some affinity for bacteria and agglutinate them in vitro. Some bacteria invading human living tissues or blood may be captured by the lymphocytes under certain conditions, although the lymphocytes have not been found to have the phagocytic function of granulocytes and monocytes.

It may well be that the extrusions contain not only agglutinating factors but also lysing factors against bacteria, because the agglutinated bacteria degenerate and become invisible more rapidly than the phagocytized bacteria. The grouping of lymphocytes in the lymph nodes, lymph follicles, lymphocytic infiltrations etc. may be a significant factor in the handling of bacteria in vivo, especially in subacute and chronic inflammations, because lymphocytes are more resistant to bacteria than other leukocytes and do not so readily degenerate into tissue debris in the living tissues.

The agglutination of bacteria by lymphocytes was more conspicuous in patients infected by homologous bacteria than in healthy controls. The same activity was, however, observed against *H. pertussis* in the blood of healthy adults and children who had had no history of whooping cough, though in lesser degree. *S. typhosa* was agglutinated by the blood lymphocytes obtained from a patient with typhoid fever, but the percentage of lymphocytes showing agglutination was much less than in the experiments with *H. pertussis*. The lymphocytes of a healthy man who had never had typhoid fever and had not been immunized against it for at least 4 years were scarcely able to agglutinate *S. typhosa*. As is well known, *S. typhosa* infecting any human body can easily invade the circulating blood and lead to bacteremia, while *H. pertussis* bacilli are usually localized in the infected sites and only rarely spread widely. It is considered that the localization of *H. pertussis* is due chiefly to the agglutinating function of lymphocytes for the following reasons: (1) The lymphocytes of healthy persons can agglutinate *H. pertussis* around themselves in vitro, whether the specific antibodies are present or not. (2) The agglutination is intensified in patients with whooping cough. (3). Absolute lymphocytosis in circulating blood begins usually in the early stage of whooping cough. (4) The enlargements of local lymph nodes and the grouping of lymphocytes and lymphogenous cells are observed in the sites of infection in animal experiments. (5) The granulocytes, monocytes and other phagocytes do not increase so much in number in the circulating blood and local tissues as do the lymphogenous cells. (6) The phagocytosis of *H. pertussis* by the phagocytic cells is generally similar to that of *S. typhosa*.

In the specimens fixed by dry-heat the agglutinated bacteria could be observed held to the surface of a lymphocyte by fine cytoplasmic extrusions which were cilia-like, and occasionally as long as 10 μ, or membranous in shape and stained light rosy pink with eosin-azure-methylene-blue solutions. Similar extrusions were also found in the specimens without bacteria. These were a special feature of lymphocytes and were scarcely seen in other leukocytes or in erythrocytes. Fine extrusions of the lymphocytes may also give forth some proteins of the cytoplasm into plasma without destruction of the cell bodies. The microscopic findings that the free ends of the extrusions are very fine and frequently disappear into the surrounding plasma support such an idea. Williamson (1950) states that rabbit lym-

phocytes extrude and shed proteins of their cytoplasm in the form of buds which contribute chemical constituents to the lymph. However, budding or clasmatosis of human blood lymphocytes was rarely seen, whereas the cilia-like or fine membranous extrusions were frequently observed (1950). If human blood lymphocytes shed their cytoplasm into the plasma, they must do so chiefly by means of these cilia-like extrusions. We should like to give the name of "roseoplasm" to the substance of these extrusions because of their color, which stains usually light rosy pink in contrast with the blue cytoplasm of the lymphocytes and probably contains agglutinating and lysing factors against bacteria.

It should be pointed out that these observations have been made in the case of a few specimens of serum; additional observations will be required to support the generalizations which have been suggested in this discussion.

SUMMARY

The blood of patients suffering from whooping cough and typhoid fever and of healthy adults and children was mixed with *Hemophilus pertussis* and *Salmonella typhosa* in vitro, incubated at 37 C, and the relationships between lymphocytes and bacteria were microscopically observed at various times.

1. The lymphocytes agglutinated *H. pertussis* around them. The phenomenon of agglutination was intensified in the patients with whooping cough.

2. The lymphocytes of the patients with typhoid fever agglutinated *S. typhosa*, but the lymphocytes of the healthy control showed scarcely any relationship with *S. typhosa*.

3. The bacteria agglutinated by lymphocytes were more rapidly dissolved than those ingested by granulocytes, especially in the ill patients.

4. The agglutinated bacteria were observed to be held to the lymphocyte by fine cytoplasmic extrusions which were peculiar to lymphocytes, and which were not basophilic. Similar extrusions of lymphocytes were also observed in simple blood smears of patients and of healthy controls without the addition of bacteria.

5. It is suggested that these extrusions may contain agglutinating and lysing factors against bacteria, and that by this means human lymphocytes may be able to capture and to dissolve bacteria, especially in the presence of homologous antibodies. The fact that *S. typhosa* often causes bacteremia whereas *H. pertussis* is usually localized in the sites of infection may be related to a difference in the affinity of the respective bacteria for lymphocytes.

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STANDARDIZATION OF A CURATIVE TEST WITH *PLASMODIUM BERGHEI* IN WHITE MICE

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The discovery by Vincke and Lips[1] of a new *Plasmodium* (*berghei*) in the Congo tree rat which is transmissible to white mice and rats as well as to other laboratory rodents provided a valuable tool for studies on biology and chemotherapy of malaria. Experimental chemotherapy of malaria before this time was limited to the use of the plasmodia of monkeys or of one of the avian malarias and involved the use of hosts which were expensive or phylogenetically far removed from man. Rodent malaria provided an experimental parasite in mammalian hosts which are well-known and inexpensive to maintain in the laboratory.

Suppressive tests for antimalarial effect of drugs, using *P. berghei* in white mice, have been standardized and used for finding the quinine equivalent of compounds by several investigators[2–4] as well as in this laboratory.[5] The absence of a good laboratory vector of *P. berghei* has prevented the development of a prophylactic test using this parasite, and consequently there is no good means of determining the effect of drugs on the primary tissue stages of the parasite. This, however, does not preclude significant investigation of the curative effect of drugs on the parasite, i.e., effect on the late tissue forms. Other laboratories have reported finding exoerythrocytic stages after blood-induced infections of *P. berghei* in white mice, and it is well-known that established blood-induced infections with avian malaria are capable of producing late tissue stages.

The course of a blood-induced infection of *P. berghei* has been shown to vary among several rodents used and according to the age of the rodent. Young white mice, white rats and hamsters develop, as a rule, an intensive parasitemia to which they ultimately succumb, while the older rodents show a varying degree of resistance to the parasite.[6] In addition to mice, rats and hamsters, other rodents reported to have been experimentally infected with *P. berghei* include meadow voles,[7] cotton rats,[8] rice rats,[7] kangaroo rats,[7] pouch mice,[7] Egyptian mice,[7] gerbils,[9] and squirrels.[10] Rabbits (except new-born) and guinea pigs appear to be refractory to the infection.[11] Our choice of young white mice for the experiments reported below is, of course, related to their small

Received for publication July 1, 1953.

This investigation was supported by a research grant from the National Institutes of Health, Public Health Service.

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drug requirements and convenience of handling in the laboratory, but chiefly to their regular susceptibility to lethal infection with *P. berghei*. This latter feature permits easy detection of most infections persisting after treatment and thus renders the mouse eminently suitable for short-term curative tests.

It has been our experience, as well as that of others,[3] that many of the suppressives for human malaria, when administered in sufficiently high doses before the parasitemia is well-established, will cure mice of blood-induced *P. berghei* infection. However, by delaying the onset of treatment after inoculation of the mice, it was found that the longer the delay before treatment was instituted, the smaller the proportion of drugs which would effect a cure. The experiments described below were designed to determine whether delay of drug administration until the infection with *P. berghei* was well-established would differentiate between those drugs having a curative effect on human vivax malaria and those having a suppressive effect.

MATERIALS AND METHODS

All mice used in these experiments were from Carworth Farms, CF_1 strain, and were young animals whose weights varied from 18 to 28 g. A single standardized dose of one million parasites of *P. berghei* (KBG 173 strain) was inoculated intravenously. Drugs were administered as a mixture in the diet at various intervals after inoculation. When drug administration was delayed, blood smears were taken on the day that treatment was started in order to ascertain that the mice in the various groups had comparable parasitemias. In experiments where the treatment was scheduled to begin on the third day after inoculation, individual mice were discarded if the parasitemia fell below 1 parasite per 1000 erythrocytes on that day. Of 372 mice inoculated with one million parasites for use in curative tests, 23 (6.2%) had a parasitemia of less than 1 parasite per 1000 erythrocytes on the third day of infection and were discarded. An additional 3 mice failed to show any evidence of parasitemia. The remainder of the 372 mice had an average of 16 parasites per 1000 erythrocytes, ranging from 1 to 56 parasites per 1000 erythrocytes on the third day after inoculation. In our experiments, all mice which developed a patent parasitemia have died unless drug therapy intervened. As a criterion of cure, blood smears were examined on the day after cessation of treatment and at intervals of 5 days thereafter for 26 days. Mice whose blood smears remained negative at the end of this time were splenectomized and were subsequently followed for 2 weeks by blood smears taken every other day, and if still negative, were considered cured. In all, 81 mice, whose blood smears were negative on the day after drug was withdrawn from the diet, proved by subsequent examination to be infected. Of these, 91% had positive blood on the second and third examinations (6 and 11 days after withdrawal of drug), and 5% were positive on the last three examinations (16, 21, and 26 days after drug withdrawal). An additional 4%, with a previous total of 6 negative blood smears, revealed positive blood following splenectomy.

RESULTS

Time relations of inoculation and treatment to curative effect.—Three drugs with a well-known curative or suppressive effect in human vivax malaria—primaquine, hydroxychloroquine, and quinine—were administered in maximum tolerated doses for periods of 5 and 10 days, beginning (a) the day of inoculation, (b) the third day after inoculation, and (c) the sixth day after inoculation. The results of this experiment, shown in table 1, confirmed the fact that delay in the onset of treatment resulted in fewer cures and showed a striking difference between the two suppressive drugs tested (hydroxychloroquine and quinine) and the known curative drug (primaquine) in curing well-established infections of *P. berghei* in mice. Treatment beginning on the day of inoculation cured most of the animals regardless of the type of drug used. Treatment begun 3 and 6 days after inoculation continued to cure most of the mice treated with primaquine (17 of 18 animals), but failed to show a similarly

TABLE 1.—*Rate of cures relative to therapeutic schedules of selected drugs.*

Drug	Period of treatment (days)	Daily average mg. drug/kg	Cure/number of mice
Primaquine	0–10	108.6	4/4
	0–5	84.1	5/5
	3–13	85.4	5/5
	3–8	72.7	4/5
	6–16	86.8	3/3
	6–11	66.0	5/5
Hydroxychloroquine	0–10	277.4	5/5
	0–5	168.6	4/5
	3–13	371.9	3/6
	3–8	183.9	0/5
	6–16	298.2	1/5
	6–11	141.3	0/5
Quinine	0–10	1021.0	3/5
	0–5	791.6	4/5
	3–13	950.6	0/5
	3–8	681.6	0/5
	6–16	795.0	0/5
	6–11	689.3	0/5

high cure rate in those groups treated with hydroxychloroquine (4 of 21 mice) and quinine (none of 20 mice).

Relative curative effects of representative 8-aminoquinolines.—The demonstration of a differentiation between representative suppressive and curative drugs when treatment was delayed led to experiments to determine the relative curative effects in such a test of three 8-aminoquinolines, whose effects in human vivax malaria are well-established. Using a standard treatment period of days 3 to 8 inclusive after inoculation, pamaquine, pentaquine, and primaquine were tested in this manner with serial drug concentrations in repeated experiments, as illustrated in table 2.

TABLE 2.—*Comparison of curative doses 8-aminoquinolines.*

Drug	Percent drug in diet	Number of mice cured/number of mice treated				
		Test				
		I	II	III	IV	Total
Pamaquine	0.09	1/4		0/5		1/9
	0.03	0/5				0/5
	0.03	0/5				0/5
Pentaquine	0.27	2/2	1/5	3/4		6/11
	0.09	2/3	2/5	0/5		4/13
	0.03	0/5		0/5		0/10
	0.01	0/5				0/5
Primaquine	0.09	4/4		3/4		7/8
	0.03	5/5		2/5	2/5	9/15
	0.01	2/5		0/5	0/5	2/15

Treatment period: days 3 to 8 after inoculation.

TABLE 3.—*Comparison of curative doses 8-aminoquinolines.*

Drug	Percent drug in diet	Daily average mg. drug/kg			
		Test			
		I	II	III	IV
Pamaquine	0.09	85.0		79.2	
	0.03	48.9			
	0.01	16.2			
Pentaquine	0.27	157.3	181.0	162.8	
	0.09	120.6	146.9	109.6	
	0.03	48.0		41.2	
	0.01	14.8			
Primaquine	0.09	91.9		78.1	
	0.03	48.1		37.8	40.69
	0.01	18.0		12.9	16.63

Actual amounts of the drugs consumed in the four tests are shown in table 3. Throughout the range of concentrations tested, primaquine had the greatest effect, pentaquine less effect, and pamaquine the least curative effect. For example, with 0.09% drug base in the diet, primaquine cured 7 of 8 mice, pentaquine, 4 of 13 mice, while pamaquine cured only 1 of 9 mice. The relative curative effects of these three 8-aminoquinolines with *P. berghei* are summarized in figure 1.

Curative effects of miscellaneous drugs.—Several drugs known to have a prophylactic or curative effect in avian malaria were tested for curative effect with *P. berghei*, using days 3 to 8 inclusive after inoculation as a treatment period. The results are shown in table 4. Endochin (SN13421), a curative and causal prophylactic drug for *P. cathemerium* in canaries, was tested and found to cure 7 of 13 mice when administered as a 0.3% mixture in the base in the diet. Lower concentrations failed to cure. Of the antibiotics tested, chlortetracycline, oxytetracycline, and chloramphenicol all have been shown to have various degrees of prophylactic activity in *Plasmodium cathemerium* although no curative effect.[5] Chlortetracycline showed some curative effect with *P. berghei* in white mice, curing 3 of 5 mice at a concentration of 0.3% drug

TABLE 4.—*Curative effect of miscellaneous drugs.*

Drug	Percent drug in diet	Mg base/ kg daily	Number of mice cured/ Number of mice treated
Endochin	0.3	482.7	7/13
	0.1	164.2	0/5
	0.03	43.5	0/5
Chlortetracycline	0.3	536.7	3/5
Oxytetracycline	1.0	1521.0	0/5
	0.3	480.3	0/5
Chloramphenicol	1.0	1180.0	0/5
	0.3	402.6	0/5
Neomycin	Subcut.	300.0	0/5
Daraprim	0.03	46.4	5/5
	0.01	15.6	4/5
	0.003	4.45	10/10
	0.001	1.6	5/5
	0.0003	0.38	0/5

Treatment period: days 3 to 8 after inoculation.

base in the diet. Oxytetracycline, chloramphenicol, and neomycin all failed to cure mice in maximum tolerated doses. Daraprim (2:4-diamino-5-*p*-chlorophenyl-6-ethyl-pyrimidine), which has a suppressive and prophylactic effect against *Plasmodium gallinaceum* in chickens as well as with *Plasmodium vivax* in man, was the most effective curative drug tested, curing 5 out of 5 mice at drug-diet concentrations as low as 0.001%.

DISCUSSION

It is apparent from the preceding experiments that a period of time for establishment of the blood-induced infection of *P. berghei* in white mice is necessary before a clear-cut difference in the cure rate becomes evident, separating drugs with a suppressive effect on vivax malaria from drugs with a curative effect. Whether this necessary establishment of an infection to demonstrate qualitative differences among drugs is due to an exoerythrocytic de-

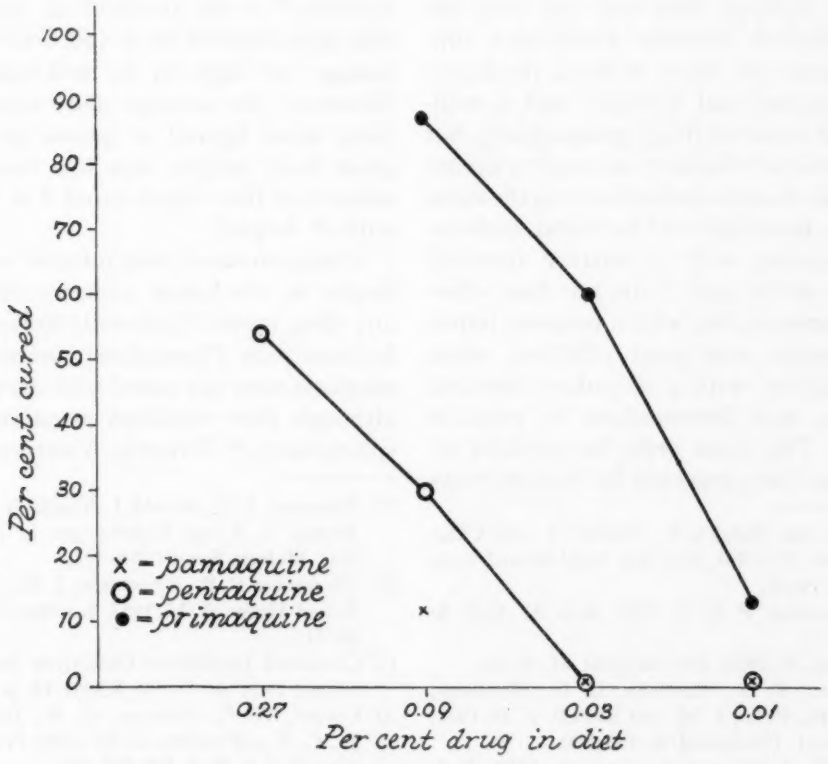

FIG. 1.—Relative curative effects of three 8-aminoquinolines with *P. berghei*.

velopment of *P. berghei* cannot be stated with the evidence at hand, but must be borne in mind as a definite possibility. Exoerythrocytic forms have been demonstrated by van den Berghe, Vincke and Chardome[12] to be present in white mice with blood-induced infections as early as 36 hours after inoculation with *P. berghei*. Garnham[13] mentions finding exoerythrocytic parasites of *P. berghei*. towards the end of the first week of the infection in white rats and hamsters after blood inoculation. Other investigators, including Baldi,[14] Falco et al,[15] and Galliard and Lapierre,[16] have failed to confirm the presence of exoerythrocytic forms in blood-induced *P. berghei* infections.

The curative test as described above, using *P. berghei* in white mice, appears to have merit in the screening of compounds for curative effect in human vivax malaria. Not only did this test differentiate between well-known suppressives for vivax malaria (hydroxychloroquine and quinine) and a well-known curative drug (primaquine), but the order of efficiency as curative agents of three 8-aminoquinolines was the same as has been reported for vivax malaria. Pamaquine, with a tertiary terminal amine on the side chain, was least effective, primaquine, with a primary terminal amine was most effective, while pentaquine, with a secondary terminal amine, was intermediate in curative effect. This same order for curative effect has been reported for human vivax malaria by Edgcomb et al.[17]

On the other hand, the only established infection of avian malaria which can be cured with an 8-aminoquinoline is *P. lophurae*.[18] Of the six 8-aminoquinolines found to have a curative action with this parasite, the compounds with secondary terminal amines as side chains proved to be most effective, while pamaquine and primaquine had moderate or slight curative effect.

Of the other drugs evaluated, which showed some curative effect in *P. berghei*, endochin has not been given trial as a curative in human vivax malaria, although a limited trial as a prophylactic in this infection did not produce a delay in parasitemia.[19]

Trials with chlortetracycline in *P. vivax* infection of man failed to cure, although there was a delay in patent parasitemia of sporozoite-induced infections.[20] In the above trials, the drug was administered for 5 and 6 days at a dosage too high to be well-tolerated. However, the average daily consumption, when figured in grams per kilogram body weight, was less than one-seventh of that which cured 3 of 5 mice with *P. berghei*.

Daraprim cured mice infected with *P. berghei* in the lowest concentrations of any drug tested. Sporozoite-induced infections with *Plasmodium cynomolgi* in monkeys were not cured with daraprim, although they remained negative until splenectomy.[15] However, Coatney[21] has

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found that in human vivax infections, 25 mg weekly for 8 weeks results in complete cure of over 50% of the cases treated.

The sulfa drugs to which *P. berghei* appears to be very susceptible have not been tried with the described curative test, and may lack correlation with the absence of curative effect in *P. vivax* infections.

SUMMARY

1. A test for curative effect of drugs on *Plasmodium berghei* in white mice has been devised and assessed as an indicator for drugs with curative effect on *Plasmodium vivax* infections in humans.

2. When drugs were administered beginning on the day of infection, a high percentage of mice were cured of *P. berghei*, regardless of whether the drug used had curative or only suppressive effect on *P. vivax*.

3. When administration of drug was delayed for 3 or 6 days after inoculation, the cure rate was markedly greater in mice receiving vivax curative drugs, as opposed to those receiving drugs with a suppressive effect on *P. vivax*.

4. The order of curative effect of representative 8-aminoquinolines was the same as found by others for human vivax infections when the drug was given on days 3 to 8 of the infection. Primaquine was the most effective, pamaquine the least effective, and pentaquine was intermediate in curative effect.

5. Of several miscellaneous compounds tested for curative effect with *P. berghei* in mice, oxytetracycline, chloramphenicol, and neomycin were ineffective at maximum tolerated doses. Endochin and chlortetracycline had some curative effect at high doses, while daraprim was the most effective drug tested and cured at levels of 1.6 mg per kg in the diet daily for 5 days.

A TECHNIQUE EMPLOYING EMBRYONATED CHICKEN EGGS FOR THE INFECTION OF ARGASID TICKS WITH *COXIELLA BURNETII*, *BACTERIUM TULARENSE*, *LEPTOSPIRA ICTEROHAEMORRHAGIAE*, AND WESTERN EQUINE ENCEPHALITIS VIRUS

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The technique of feeding blood-sucking arthropods on chicken embryos has been used for the transmission of microbial diseases. Haas and Ewing (1945), for example, transmitted *Plasmodium gallinaceum* to chicken embryos by feeding *Aëdes aegypti* through the shell membrane. Ferris and Hanson (1952) described a technique of feeding mosquitoes and flies on embryonated chicken eggs. Without giving any results they also reported the feeding of these insects through the air-cell membrane of normal and infected eggs in experiments on the transmission of vesicular stomatitis virus.

In 1949, one of the authors (E.G.P.), while engaged in studies involving the infection of ixodid ticks with spotted fever rickettsias, made several unsuccessful attempts to feed *Dermacentor andersoni*, *Amblyomma maculatum*, and *Amblyomma americanum*, as well as the mite *Bdellonyssus bacoti*, on the air-sac membrane of chick embryos. More recently, however, in connection with studies of the behavior of pathogenic microorganisms in argasid ticks, it was found that several species of the genus *Ornithodoros* fed successfully on the air-sac membrane of normal eggs and of eggs infected with *Coxiella burnetii*, *Bacterium tularense*, *Leptospira icterohaemorrhagiae* and western equine encephalitis virus. The method employed and results obtained in these experiments are the subject of this paper.

Received for publication August 5, 1953.

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MATERIALS AND METHODS

All ticks used in the following experiments were reared at the Rocky Mountain Laboratory. The ticks studied included *Ornithodoros moubata* (Africa), *turicata*, *parkeri*, *hermsi* (western United States), and *Argas reflexus* (western United States). The fertile hens' eggs were incubated at 101 F for 10 to 12 days. The shell above the air sac was removed by means of a dental carbon disc, and the ticks placed on the shell membrane overlying the chorioallantoic membrane (see fig. 1). The eggs were then sealed tightly with cellulose tape and returned to the incubator for at least 8 to 12 hours. The adherence of ticks (especially larvae and nymphs) to the tape can be prevented by applying a piece of gauze over the opening before it is sealed.

The data in table 1 show that a varying number of ticks of each species, except the bird tick, *A. reflexus*, fed upon the embryonated eggs. The highest percentage of engorged ticks belonged to *O. turicata* (72 of 80) and *O. moubata* (130 of 195). If the eggs, containing *O. moubata*, were taken out of the incubator after 12 hours and placed at room temperature for 2 to 3 hours with the cellulose tape removed, an increase to almost 100% of engorged ticks could be obtained. Attempts to induce *O. hermsi*, *O. parkeri*, and *A. reflexus* to feed by the placing of droplets of human saliva on the membrane did not increase the numbers of fed ticks. Stained smears of gut contents, obtained shortly after the ticks had been removed from the eggs, revealed that the ticks had ingested chicken blood.

The successful results of the described feeding experiments indicated that this method might be employed as a practical means of infecting argasid ticks with certain pathogenic microorganisms which grow well in egg cultures. Accordingly, we fed *O. turicata* as well as *O. moubata*

TABLE 1.—*Results of feeding argasid ticks on the air-sac membrane of normal eggs.*

Tick species	Egg no.	Number of ticks placed on air-sac membrane	Fed	Percent
O. turicata (4th nymphal stage)	1	10	8	80
	2	10	10	100
	3	10	9	90
	4	10	10	100
	5	10	7	70
	6	10	9	90
	7	10	9	90
	8	10	10	100
Total		80	72	90
O. moubata (4th nymphal stage)	9	10	8	80
	10	20	17	85
	11	30	25	83
	12	35	17	48
	13	50	20	40
	14	50	43	86
Total		195	130	67
O. hermsi (4th nymphal stage)	15	10	2	20
	16	10	7	70
	17	10	8	80
	18	15	6	40
	19	15	0	0
	20	20	14	70
	21	20	5	25
Total		100	42	42
O. parkeri (adult)	22	10	0	0
	23	10	6	60
	24	10	4	40
	25	15	9	60
	26	15	4	27
Total		60	23	38
A. *reflexus* (adult)	27–36	10 on each egg	0	0
Total		100	0	0

on embryonated eggs infected with *C. burnetii*, *Bact. tularense*, *Lept. icterohaemorrhagiae* and western equine encephalitis virus. The results of these experiments are briefly reported in the following paragraphs.

RESULTS

1. *The infection of* O. moubata *with* C. burnetii

Sixty 5-day-old chicken eggs were inoculated with 0.5 ml of yolk-sac material infected with the Nine Mile strain of Q fever (Davis and Cox, 1938). The dosage used killed 42 of the embryos by the 7th day postinoculation. On the 6th day 200 *O. moubata* of the third nymphal stage were placed on the air-sac membrane for feeding. Eighty of these ticks had engorged after 12 hours and were stored at room temperature for titration tests in guinea pigs.

Seven, thirty-five, and eighty-seven days after the infective feeding, lots of 10 ticks each were washed in 1:1000 aqueous merthiolate for 15 minutes, rinsed in saline to remove the disinfecting merthiolate, and triturated in 10 ml buffered saline. The suspensions were used as starting material for dilutions 10^{-1} through 10^{-6} in experiment 1 (with ticks infected 7 days previously), 10^{-3} through 10^{-6} in experiment 2 (with ticks infected 35 days previously), and 10^{-5} through 10^{-10} in experiment 3 (with ticks infected 87 days previously). One ml of each dilution was inoculated intraperitoneally into four guinea pigs. In experiment 1, the guinea pigs injected with dilutions up to 10^{-3} and in experiments 2 and 3 those injected with dilutions up to 10^{-7} developed positive complement-fixing antibodies against *C. burnetii*. These results prove the ability of *O. moubata* to ingest the rickettsias by feeding on infected chicken embryos. They also confirm the development and persistence of *C. burnetii* in this tick, as reported by Davis (1943), Weyer (1948, 1949, 1950), Burgdorfer (1951) and Jadin (1951).

2. *The infection of* O. moubata *and* O. turicata *with* Bact. tularense

Seven-day-old chicken eggs were infected with *Bact. tularense*, strain no. 14, isolated in 1951 from a cottontail rabbit, *Sylvilagus* sp., in Nevada. The ticks were placed on the air-sac membrane on the 5th day postinoculation, when 50% of the inoculated eggs were dead due to the infection. Eighty-four of 100 *O. moubata* and 49 of 50 *O. turicata* became engorged. To demonstrate infection in these ticks, two pools of each species were titrated in mice 6 days after the infective feeding. After the ticks had been washed with merthiolate and rinsed in saline, three per pool were ground in buffered saline (1/10 ml per

mg of tick weight). The suspensions were used as starting material for dilutions 10^{-5} through 10^{-12}, and six 21-day-old mice were injected subcutaneously with 0.2 ml of each dilution. In all four experiments, high concentrations of *Bact. tularense* were found in the tick tissues, with no differences noted between the two tick species (see table 2).

In transmission studies, the course of infection was also followed by means of smears and microscopic sections. So far it has been found that *Bact. tularense* not only persists and develops in the ticks for a period of more than 200 days but that it is also ejected with the coxal fluid during the feeding of the ticks.

TABLE 2.—*Titration of* O. moubata *and* O. turicata *fed upon* Bact. tularense-*infected chicken eggs.*

Tick species	Pool no.	Dilutions containing one mouse LD_{50}*
O moubata	1	$10^{-9.74}$
	2	$10^{-9.50}$
O. turicata	1	$10^{-9.50}$
	2	$10^{-9.74}$

* Logarithm of 50% infective end point, as determined by the method of Reed and Muench (1938).

3. *The infection of* O. moubata *and* O. turicata *with* Lept. icterohaemorrhagiae

A description of the experimental infection of argasid ticks with leptospiras has not been recorded in the literature.* The fact that leptospiras had been recovered from rodents which serve as hosts of ticks and other arthropods suggested the feeding of *O. moubata* and *O. turicata* on eggs infected with *Lept. icterohaemorrhagiae* to determine if these ticks might serve as vectors of leptospiras.

Seven-day-old chicken eggs were injected with 0.4 ml of a leptospira culture in Verwoert's medium, strain 204, isolated from rats and maintained at the Rocky Mountain Laboratory. On the 4th day postinoculation, 72 of 80 *O. moubata*, fourth nymphal stage, and 56 of 64 *O. turicata*, fourth nymphal stage, fed on the infected chicken eggs.

Each day after the feedings, ticks were dissected and their gut contents examined for the presence of living leptospiras by the darkfield technique. Active leptospiras were found easily during the first 3 days of infection but not in examinations made after this time.

Seventeen days after the feeding on infected eggs, four ticks of each species were separately ground up in 2.5 ml of saline and injected intraperitoneally into two guinea pigs. The suspensions of *O. moubata* did not produce fever, but the animals injected with the suspensions of *O. turicata* became febrile on the 5th day, and the blood of these guinea pigs, cultivated on Chang's medium, revealed growth of *Lept. icterohaemorrhagiae* after 5 days.

Twenty-three days after the infective feeding, *O. moubata* were again tested for the presence of leptospiras pathogenic for guinea pigs. A lot of 10 ticks was ground in 3.0 ml of saline, and 1.0 ml of the suspension was inoculated into each of two guinea pigs. Both animals developed fever on the 4th day postinoculation, and the presence of leptospiras in their blood was proved by cultivation in Chang's medium. These results indicate the persistence of the bacteria in at least some ticks.

After the survival of leptospiras in *O. turicata* as well as in *O. moubata* had been established, the site of its persistence was investigated. Intensive studies on this question are in process. To date it has been found that the

* After this paper had been accepted for publication, it was learned that Schlossberger and Langbein (1952) had reported the experimental infection of *O. moubata* with *Lept. icterohaemorrhagiae* after feeding on infected guinea pigs.

leptospiras penetrate the gut wall, enter the body cavity, and invade principally the central ganglion and the coxal organs; they also are ejected with the coxal fluids during the tick feeding.

4. *The infection of* O. moubata *and* O. turicata *with western equine encephalitis virus*

Blood-sucking arthropods, mosquitoes, and ticks are responsible for the transmission and distribution of several neurotropic viruses, such as equine encephalitis, St. Louis encephalitis, and louping ill. Syverton and Berry (1937) reported the successful experimental infection of *D. andersoni* with equine encephalomyelitis and demonstrated the survival of the virus through all tick stages, including the eggs. No work has been published, however, in which ticks of the genus *Ornithodoros* were examined to determine their ability to serve as vectors of such diseases. As ticks of this genus have been shown to feed readily on eggs, attempts were made to infect *O. moubata* and *O. turicata* with western equine encephalitis virus. In these experiments, 10-day-old eggs were injected with 0.05 ml of an inoculum which was made of infected chicken embryos and which killed 75 of 85 eggs within 24 hours. A total of 84 of 100 *O. moubata* and 27 of 30 *O. turicata*, all in the fourth nymphal stage, engorged upon the eggs.

Fifteen days after the infective feeding, 4 ticks of each species were ground in 1.5 ml saline (containing 10% rabbit serum, 450 units of penicillin, and 450 mg streptomycin), and 0.05 ml of this suspension was injected intraperitoneally into six 6-day-old mice. As a control, suspensions of normal ticks were injected into other litters.

All mice which received the suspen-

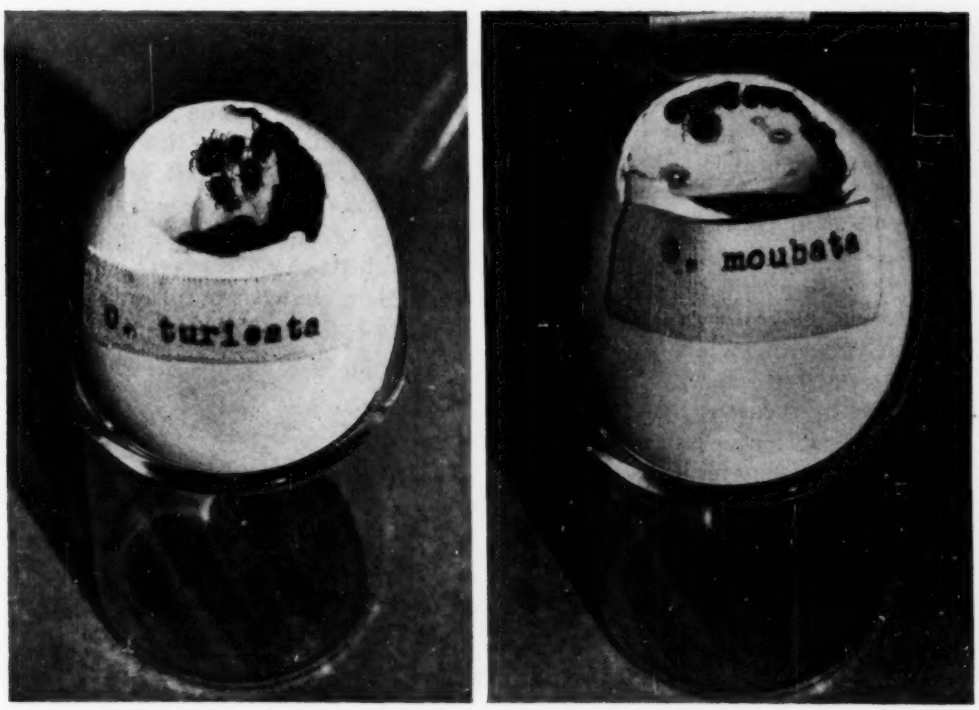

FIG. 1.—*O. moubata* and *O. turicata* feeding on the air-sac membrane of embryonating chicken eggs.

sions of *O. turicata* died within 48 hours after the inoculation. Those injected with the suspensions of *O. moubata* and of clean ticks—*O. moubata* as well as *O. turicata*—remained normal. Twenty-five days after feeding, a second test was performed with 6 *O. moubata* and 3 *O. turicata*. All mice of this experiment were dead on the 3rd day. In order to prove that the death of these mice was due to the viral infection, the brains were triturated in normal rabbit serum, and the suspensions were set up against immune serum of rabbits which had been infected with a well-known strain of western equine encephalitis. The positive results of these neutralization tests proved the infectivity of the ticks for mice and also the survival of the virus in the ticks for at least 25 days.

5. The detection of infected argasid ticks by feeding on normal eggs

The technique of feeding ticks on embryonated chicken eggs is useful not only for infecting ticks with various agents which may be cultivated in chicken embryos, but also for the detection of infected ticks. An example is given in the following transmission experiments with relapsing fever spirochetes, *Borrelia turicatae* and *Borrelia parkeri*.

Experiment 1.—Twenty *O. turicata*, the progeny of a female infected with *B. turicatae* and therefore probably also carriers of spirochetes by transovarial infection, were fed in groups of 5 ticks each on four 11-day-old normal chicken eggs. After 5 days incubation at 96 F, the embryos were examined for the presence of spirochetes. The occurrence of *B. turicatae* in three eggs indicated the infectivity of and transmission by the ticks.

Experiment 2.—One *O. parkeri* infected with *B. parkeri* was fed on an 11-day-old normal egg. The presence of spirochetes in the blood of the chicken embryo indicated infection from the tick.

DISCUSSION

Many epidemiological problems in relation to the distribution and transmission of human as well as animal diseases by blood-sucking arthropods are still unsolved, and the life cycle of pathogenic microorganisms in different vectors is the subject of intensive studies. Little, for instance, is known about the importance of arthropods in the epidemiology of leptospirosis; this in spite of the fact that leptospiras have been recovered from numerous rodents which may serve as hosts of such ectoparasites. Another problem of interest is the question of whether argasid ticks in addition to ixodid ticks and insects play a significant role in the maintenance and distribution of virus diseases in nature. Many of the agents (rickettsias, leptospiras, viruses, and others) are cultivated in embryonated chicken eggs for experimental work. The described technique of feeding argasid ticks on the air-sac membrane of eggs represents, therefore, a practical and economical method not only of infecting ticks in order to study the behavior (persistence, development, change of virulence, etc.) of pathogenic microorganisms but also of identifying vectors and transmitters of diseases.

SUMMARY

1. A technique for feeding argasid ticks of the genus *Ornithodoros* (*O. moubata*, *O. turicata*, *O. parkeri*, *O. hermsi*) on the air-sac membrane of embryonated chicken eggs is reported. Several attempts to feed *Argas reflexus* by the same method failed.

2. The technique is also practical for testing argasid ticks for their ability to

ingest, to preserve, or to transmit disease agents that can be cultivated in eggs and also for research studies regarding the life cycle and behavior of such agents in ticks.

3. Infections of *O. moubata* and *O. turicata* with *Coxiella burnetii*, *Bacterium tularense*, *Leptospira icterohaemorrhagiae* and western equine encephalitis virus by feeding on infected eggs were successfully accomplished.

4. In transmission studies, normal eggs were used to detect infections of relapsing fever spirochetes in *O. turicata* and *O. parkeri* ticks.

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VIRULENCE OF THE TUBERCLE BACILLUS

I. EFFECT OF OXYGEN TENSION UPON RESPIRATION OF VIRULENT AND AVIRULENT BACILLI

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An adequate explanation of the factors underlying the virulence of the tubercle bacillus is not yet available although a number of chemical, metabolic and morphologic characteristics have been found to correlate well with the disease-producing capacity of the organism.[1] These include the presence in the bacillary cell of certain chemical constituents,[2–7] the activities of bacilli or their components in various chemical,[8–12] physical[13] and biologic tests,[5,6,14–16] the differential influence of various substances upon the oxygen uptake or growth of virulent as compared with avirulent bacilli,[17–24] the orientation of bacterial cells in developing colonies[25–27] and their acidfastness,[26] and the readiness of bacillary growth in culture mediums.[25–27] Whether any one of these features directly accounts for the fact of progressive multiplication of virulent bacilli in a susceptible host is not yet settled. In certain instances the relationship of these features to viru-

Received for publication August 8, 1953.

This work has been supported by grants from the National Tuberculosis Association, the California Tuberculosis and Health Association, and the Alameda County (California) Tuberculosis and Health Association, and in part by a research grant E-110 (C_3) from the National Microbiological Institute, of the National Institutes of Health, Public Health Service.

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lence is very suggestive, as will be detailed below. However, the property of virulence may be predicated upon two broad bases; the first, that a microbe possesses the capacity to overcome in some way the native defensive abilities of the host and thus in an aggressive fashion to establish itself in the tissues; the second, that the parasite needs no special qualifications except those metabolic and nutritional characteristics required to utilize the host environment as a growth medium.

The first case is exemplified by virulence of the pneumococcus. The leucocytes of the normal host are potentially protective against this organism, but ordinarily the virulent pneumococcus successfully evades engulfment by these cells, an ability which it owes to its polysaccharide capsule. Hence the pneumococcal capsule, by discouraging phagocytosis, qualifies as an aggressive factor of virulence. Pneumococci without capsules are readily ingested and destroyed by normal leucocytes, and are avirulent.

The second case postulated above is illustrated by the studies of Bacon et al[28] with purine-requiring mutants of *Salmonella typhosa* and of Garber and co-workers[29] with similar mutants of *Klebsiella pneumoniae*. In both cases these mutants of virulent strains fail to proliferate in the peritoneal cavities of mice because of the unavailability of purines in that locale. In the experiments with *K. pneumoniae* it was found that injection of purine into the peritoneal cavity permitted bacillary growth and consequent death of the host. In both these cases strains of bacteria otherwise apparently equivalent to the virulent parent strain were distinguished from it by a single nutritional requirement, and on this basis lacked virulence. When this nutritional requirement was artificially supplied in the host environment, the organisms displayed virulence.

Turning to a consideration of the tubercle bacillus in the light of these examples, we find that certain of the evidence referred to earlier can be marshalled for the case of aggressive pathogenicity. In the first place, macrophages of the normal host have some capacity to inhibit bacillary growth. Virulent tubercle bacilli ingested by the normal macrophages of a susceptible host species proliferate relatively slowly, while avirulent bacilli either fail to multiply[30,31] or do so only in limited degree and eventually disappear completely from the tissues.[32] Suter[33] has reported that in tissue cultures macrophages inhibit the multiplication of avirulent bacilli. This effect is not a striking one when compared with the destructive activity of leucocytes upon pneumococci.

As for aggressive activities on the part of the parasite, there are certain indications that virulent tubercle bacilli may combat the defensive activities of macrophages. It is apparent that these organisms do not interfere with phagocytosis itself, for virulent bacilli are as readily phagocytized as are avirulent strains. Whatever antiphagocytic powers the virulent bacillus may possess must presumably come into play after the parasite has been engulfed. Bloch[5] has suggested that the cord factor acts

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as a "cover" protecting bacteria from digestion by phagocytes. A destructive activity upon polymorphonuclear phagocytes has been described by the same author[34] from experiments in mice, and by a number of other workers from studies of infected tissue cultures. Maximow,[35] Haagen[36] and others,[37] amongst earlier workers, and more recently Fell and Brieger,[38–40] Mackaness[41,42] and Suter[33] have found that virulent bovine and human tubercle bacilli disrupt the phagocytes of susceptible species in tissue culture. If this evidence were entirely clear there would be good reason to conclude that the distinctive feature of virulence of the tubercle bacillus lies in its power to injure or kill those cells of the normal body which might otherwise hinder its proliferation in the tissues. However, several considerations becloud the issue:

First, not all virulent varieties of the bacillus disrupt cells of susceptible hosts in tissue culture. Virulent avian bacilli establish a symbiotic relationship with phagocytes.[40,43–46]

Second, in at least one instance even avirulent bacilli appear to overgrow and destroy phagocytes in tissue culture; this is described as occurring with avirulent human bacilli in rabbit cell cultures.[40,47]

Third, and perhaps most important, it seems improbable that the excessive bacillary proliferation which leads to cell destruction in cultures represents the usual situation in vivo. Brieger, Fell and Smith[39,40] infected rabbits with bovine bacilli and explanted their splenic tissue to cultures. These explants were examined simultaneously with the spleen which had remained in situ, with strikingly different results. After ten days, when the spleen cultures teemed with bacilli and a good deal of phagocytic destruction had occurred, only very few organisms were demonstrable in the spleen itself, and the cells containing these were apparently healthy. Brieger and his associates suggest that this difference may owe to the presence of an inhibitory factor in vivo which is absent in tissue cultures, or, alternatively, that body cells contain organisms which are not made recognizable by the usual staining procedures.[45,46] Lurie[48] infected macrophages with virulent bacilli and transferred them to the anterior chambers of rabbit eyes. The bacteria multiplied, but here again the cells which harbored them remained alive and apparently normal.

It seems to follow from these observations that the macrophage-damaging activity of virulent bovine and human tubercle bacilli becomes manifest only after considerable intracellular bacillary proliferation has occurred, and that this occurrence is much more striking under the artificial condition of tissue culture

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than of the body itself. Whether the observations so far made can adequately explain the ability of virulent bacilli to establish themselves in the body seem, therefore, open to question.

Since the inhibitory influence of phagocytes upon avirulent bacilli seems to be a rather mild one in the sense that it does not result in an abrupt destruction of organisms, this influence is perhaps as readily explainable by failure of the interior of the cell to provide a proper milieu for growth of the microbes as upon the presence of a more specific antimicrobial mechanism.[49] As there is, furthermore, no conclusive evidence that the antiphagocytic activity of virulent bacilli observed in tissue cultures necessarily portrays the situation which exists in vivo, we are justified in questioning the importance of the part played by the "aggressiveness" of these bacilli in relation to their virulence. These doubts provide an ample basis for seeking additional evidence on the question of the nature of the virulence of this bacterium.

We are led then to the second of the general concepts of virulence alluded to above—that a major difference between virulent and avirulent tubercle bacilli may be determined by some metabolic characteristic which permits virulent organisms to better utilize the host cell as a growth medium. This suggestion has been made before,[49] and on such a premise Holmgren and Youmans[23] recently investigated the influence of various plasma constituents and of vitamins upon the generation times of virulent and avirulent bacilli. Certain substances, of which bovine serum was the best example, were found to stimulate the proliferation of virulent organisms appreciably while having no effect upon avirulent strains. These studies suggest that virulent organisms may make better use than do avirulent ones of constituents of the blood, and conceivably this may explain to some extent the ability of the virulent organism to multiply in the body. It does not, however, explain the suppression of avirulent bacilli which occurs in vivo.

It is well known that the tubercle bacillus requires oxygen for its growth and even its survival, and that the respiration and proliferation of the organism diminishes with decreasing oxygen tension in the environment.[50–58] Vorwald[59] studied the relative susceptibilities of adult and fetal guinea pigs to tuberculous infection and found the refractoriness of the latter to change to a state of receptiveness following birth. The suggestion was made that the inhospitality of the fetal tissues to the bacillus may depend upon the low oxygen tension prevailing in them. The partially inhibitory effect of low environmental oxygen tensions upon the progression of tuberculosis in animals has been demonstrated directly by Rich and Follis.[60] Olson and co-workers[61] have found that manipulation of the

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pulmonary circulation in monkeys results in more pronounced tuberculous lesions in the areas receiving more highly oxygenated blood. Recently reported studies of resected lung specimens indicate that microscopically intact bacilli from closed cavities frequently fail to grow in cultures or animals. The major basis for this may be the inhibitory or toxic effect of oxygen deficiency in these cavities.[49,57,62,63]

It seemed possible to us that a low level of oxygen might be even more repressive to avirulent organisms than to virulent, and that an oxygen tension approximating that of the tissues might reveal a differential inhibition of the activities of these two kinds of tubercle bacilli. This possibility was studied both with respect to respiration and growth. The present paper details the influence of diminished oxygen tension upon respiration, and the succeeding report documents its effects upon the multiplication of virulent and avirulent strains.

MATERIALS AND METHODS

Ten strains of *Mycobacterium tuberculosis* were used in this study. The virulent strains are designated as H37Rv, Ravenel, Campbell and Cutter; attenuated ones as BCG and R1Rv; and avirulent strains as H37Ra, JH6Ra, JH16Ra and R1Ra. Most of these are well-known standard strains which were obtained from the Trudeau Laboratory; the Cutter strain is an old laboratory culture of a human variant, which was supplied by the Cutter Laboratories of Berkeley, California. This organism was obtained from the Department of Agriculture. The BCG was obtained several years ago from Dr. Aronson of the Phipps Institute. With the exceptions of the bovine strains BCG and Ravenel, all are human varieties. The organisms were cultured at 37 C in thin layers of Dubos-Davis "Tween-albumin" medium contained in Roux bottles. The cells were harvested from the medium by centrifugation; in some experiments they were resuspended in fresh Dubos-Davis medium, while in others the cells were washed three times by centrifugation and resuspended in $M/15$ phosphate buffer of pH 6.2. The most critical detail was the preparation of fairly uniform cell suspensions of identical turbidities for each comparative test. These determinations were made with the Klett-Summerson photoelectric colorimeter, using a no. 54 filter. Clumping was minimal in the Dubos-Davis medium, and all turbidity measurements were made in it. Cell turbidity values reported in this paper represent Klett values for 1:10 dilutions of the cell suspensions employed. When phosphate buffer was the suspending fluid, turbidity measurements were made on the original culture before washing, care being taken to retain all cells in the washing procedure.

Conventional Warburg manometry, generally using a Lardy-type apparatus, was employed at 37 C. In some comparative experiments the vessels were horizontally shaken in an Aminco apparatus but no difference in results was noted. Gas mixtures were prepared by diluting air with nitrogen over water, or were delivered directly from a commercially prepared tank mixture of 1% oxygen, 0.03% carbon dioxide and 98.97% nitrogen. The Warburg vessels were flushed 10 to 15 minutes with the desired gas mixture.

The respiration experiments were designed to test pairs of strains, i.e., a virulent versus an avirulent strain, a virulent against an attenuated, or an attenuated versus an avirulent strain. Inasmuch as avirulent strains generally multiply more slowly than virulent ones, the harvest time was controlled by the extent of growth of the avirulent strain. A pair was always harvested at the same culture age, usually 9 to 14 days.

It was desired to determine whether tissue or leucocyte juices or the serum of guinea pigs might influence the respiratory activities of these bacilli at the oxygen tensions employed. Accordingly, livers, lungs and spleens from normal animals were washed well with saline and homogenized in a Waring blendor. The homogenized material was then centrifuged at 18,000 r.p.m. and the supernatant fluid collected and employed as the tissue extract. Suspensions of phagocytes were obtained from the peritoneal cavities of aleuronat-treated guinea pigs, and the particulate matter sedimented by light centrifugation was macerated with glass in a mortar. This material was further disintegrated by high speed mechanical shaking in the presence of small glass beads. The glass was removed by centrifu-

and Mattern, C. A. 1952, Am. Rev. Tuberc. **65**: 48.
62. Beck, F. and Yegian, B. 1952, Am. Rev. Tuberc. **66**: 44.
63. Medlar, E. M. 1952, Am. Rev. Tuberc. **66**: 381.

gation and the supernatant material was mixed with an equal volume of saline. Serums were obtained in the conventional manner. The respiratory activities of the different strains were determined with the cells suspended in the cellular extracts or in serum.

RESULTS

Effect of cell concentration on respiration.—In preliminary studies concerned with the determination of suitable concentrations of bacteria to be employed, it became apparent that the amounts of oxygen consumed were not proportional to the concentrations of cells employed. These observations are summarized in table 1. In no instance was the rate of oxygen consumption excessive for Warburg studies. Doubling or quadrupling the concentrations of bacteria in the Warburg flasks, all other factors being constant, did not result in corresponding increases in the amounts of oxygen consumed, and the departure from a linear relationship was much more pronounced at the lower oxygen tension. The greatest difference in the extent of respiratory inhibition at the low oxygen tension as between avirulent and virulent bacilli occurs at the lowest cell concentrations. With increase in cell population, the inhibitory effect of the low oxygen tension upon the respiration of the virulent strain approaches that observed with the avirulent one. These results, in part, reflect peculiarities of the tubercle bacillus and, to a lesser extent, limitations of the Warburg technique. These studies, and other preliminary ones with *Myco. tuberculosis*, indicate that the concentrations of cells in comparative studies of the respiration of virulent and avirulent bacilli must be as nearly the same as possible. In earlier studies in this laboratory Futrelle[64] and Marino[65] had observed that the rates of respirations of various other bacteria were not directly proportional to the concentrations of the cell suspensions, all other variables being constant. Discrepant results reported in the literature may in part be due to failure to recognize this effect of cell concentration.

Effect of oxygen tension upon respiration.—Turning now to the question of oxygen utilization under reduced oxygen tension, Kempner[55,66] concluded, contrary to the generally accepted concept, that the rate of respiration of various cells is dependent upon oxygen tension. Its influence was found to be more pronounced at 37 C than at temperatures close to 0 C, the solubility of oxygen being considerably less at the higher temperature. In studies in this laboratory[64,65] it has also been observed with various bacteria that the rate of respiration depends upon the oxygen tension. At constant cell concentrations these results are in agreement with those of Kempner, i.e., the rate of respiration decreases below limiting oxygen tensions characteristic for a given species and test conditions.

In our studies with *Myco. tuberculosis* the vessels were flushed with the gas mixture under test at least every 30 minutes when the oxygen consumption was such that a marked change in tension occurred. Oxygen consumption values per hour are calculated from periods of observation of 30 minutes or less to reduce the error introduced by marked change in tension during the measurements. The results, as indicated by the figures mentioned earlier for the amounts of oxygen consumed by the 1:4 dilution of the stock suspension, reveal

64. Futrelle, C. M. 1951, Influence of Oxygen Tension on the Respiration of the Tubercle Bacillus, Master of Arts Thesis, Stanford University.

65. Marino, R. J. 1952, Oxygen Tension, Cell Concentrations and Bacterial Respiration, Master of Arts Thesis, Stanford University.

66. Kempner, W. 1939, Cold Spring Harbor Symposium on Quant. Biol. 7: 269.

that the oxygen demand of even the most dilute cell suspension is not met under the experimental conditions when the cells are respiring in an atmosphere containing 1% oxygen. The oxygen in the vessel is not completely utilized, approximately 150 mm^3 having been present at the start and slightly more than 100 mm^3 at the end of 1 hour. When this matter was further examined by testing the same volumes of cell suspensions in larger Warburg flasks of 40 and 125 ml capacity, in which the total oxygen available and its rate of diffusion were increased, less respiratory inhibition was noted, but the sensitivity of the measurements was greatly reduced. These studies suggest that the rate of oxygen utilization may actually be independent of the oxygen tension down to very low levels of oxygen under ideal conditions. But these conditions are seldom encountered in ordinary manometric studies, and certainly would not prevail in vivo.

Comparative respirations of virulent, attenuated and avirulent bacilli under low oxygen tension.—As stated under *Methods*, cells from cultures of the same chronological age were employed in the comparative studies. The growth of virulent strains generally occurs at a more rapid rate than that of avirulent ones, and it could be argued that physiological age of the cells might influence the results. Comparative studies were made with cells from cultures of different ages, and little or no difference could be noted in their respiratory activities. In one or two instances H37Ra and H37Rv gave approximately the same crop yield from inoculums of similar size in the same length of time. The relative behavior of these cells was the same as that of cells from cultures of the same age but in which H37Rv gave much higher crop yields than H37Ra. Physiological and chronological ages were the same in the first case, probably different in the latter, but respiratory differences were the same as far as could be ascertained. It appears, therefore, that the major variable is cell concentration, and this was rigidly controlled. When the rate of respiration was relatively high at the low oxygen tension, the flasks were flushed as mentioned above with the gas mixture at appropriate intervals so that, in general, the oxygen tension in the flasks was maintained at a level of 0.5 to 1.0%. Typical results of oxygen consumption studies of cells of the various strains suspended in phosphate buffer, in the buffer plus lactate as the substrate, and in Dubos-Davis medium are presented in table 2.

TABLE 1.—*Influence of cell concentration on the inhibition of respiration of tubercle bacilli at reduced oxygen tension.*

Strain	Dilution	Air*	1% O_2*	Percent inhibition
H37Ra		208	50	76
	1:2	88	31	65
	1:4	59	35	41
H37Rv		126	41	67
	1:2	72	47	35
	1:4	57	45	21

* Mm^3 oxygen consumed in 60 minutes in Dubos-Davis medium.

There are two quite consistent features evident in these data: first, the avirulent strains tend to respire at a higher rate than do their virulent test partners of the same turbidity; and second, the respiration of an avirulent strain is inhibited to a greater extent than that of its virulent partner at an oxygen tension of 1%. Essentially the same behavior was noted when acetate was substituted for lactate as a substrate. In some instances the difference between the extent of inhibition of the avirulent and the virulent member of a pair was not pronounced but the tendency for the avirulent partner to be inhibited to a greater extent was always observed in these and in similar experi-

TABLE 2.—*Respiration of virulent, attenuated and avirulent strains of tubercle bacilli in phosphate buffer, pH 6.2, and in Dubos-Davis medium.*

Strain	Endogenous			Lactate			Dubos-Davis		
	Air*	1% O_2*	Percent inhibition	Air*	1% O_2*	Percent inhibition	Air*	1% O_2*	Percent inhibition
H37Ra (AV)	129	35	73	212	66	69	132	30	76
H36Rv (V)	106 [285]†	32	70	165 [350]	61	63	87 [285]	22	75
BCG (AT)	96	58	40	117	49	59	148	58	61
Ravenel (V)	66 [335]	47	29	74 [260]	41	45	81 [335]	53	35
JH6Ra (AV)	31	15	52	317	70	78	90	30	66
Campbell (V)	16 [147]	11	31	232 [450]	66	72	50 [147]	26	48
JH16Ra (AV)	52	25	52	201	74	63	118	48	59
Cutter (V)	48 [190]	27	44	99 [380]	63	36	95 [190]	44	53

AV = avirulent, V = virulent, and AT = attenuated strain.
* Mm^3 oxygen consumed in 60 minutes.
† The figures in brackets are Klett readings on 1:10 dilutions of the suspensions employed in each comparative test.

ments. The concentration of the cells and the nature of the test strain influence the numerical values of the results.

In other experiments the cells were suspended for the manometric measurements in normal serum, tissue juice or leucocyte extract to provide chemical constituents in the medium more closely approaching those encountered in the host animal. The results of oxygen consumption studies with serum or with tissue juice as the suspending medium are reported in table 3. The pattern of respiratory activity is entirely similar to that shown in table 2, the avirulent strain respiring more actively than its virulent test partner in an atmosphere of air and being inhibited to a greater extent at 1% oxygen tension. The same behavior was noted when the cells were suspended in leucocyte extract, but this phase of the study was limited to the pairs H37Ra-H37Rv and BCG-Ravenel. With the former pair inhibitions of 45 and 25%, respectively, were noted, with the latter 43 and 40%. Similar results, to be reported elsewhere, were obtained when the cells were suspended in serums or in tissue extracts from immunized animals.

DISCUSSION

The results recorded in this paper are suggestive rather than conclusive; they indicate that the respiration of avirulent strains of the tubercle bacillus is inhibited to a greater extent at 1% oxygen tension than is that of virulent strains. The endogenous oxygen consumption values reported in table 2

TABLE 3.—*Respiration of virulent, attenuated and avirulent strains of tubercle bacilli in the presence of normal serum or of tissue juice.*

Strain	Turbidity†	Serum			Turbidity†	Tissue juice		
		Air*	1% O_2*	Percent inhibition		Air*	1% O_2*	Percent inhibition
H37Ra (AV)	208	201	85	58	260	96	21	78
H37Rv (V)	208	116	75	35	260	83	24	71
BCG (AT)	160	219	76	65	220	110	43	60
Ravenel (V)	160	158	88	45	220	69	31	55
JH6Ra (AV)	187	261	69	74	170	72	40	44
Campbell (V)	187	103	53	49	170	55	33	40
JH16Ra (AV)	415	218	62	72	245	131	46	65
Cutter (V)	415	166	51	69	245	94	37	61
R1Ra (AV)	210	167	70	58	235	105	31	70
R1Rv (AT)	210	88	61	32	235	93	38	59

AV = avirulent, V = virulent, and AT = attenuated strain.
* Mm^3 oxygen consumed in 60 minutes.
† Klett readings on 1:10 dilutions of the suspensions employed.

show a range of respiratory inhibition at 1% oxygen tension of from 29 to 70% for virulent strains and of 52 to 73% for avirulent ones. Offhand, this suggests that certain avirulent strains are inhibited to a lesser extent than are some virulent ones. However, such a comparison is probably not valid, for each strain exhibits a different Q_{O_2}, and the effect of cell concentration differs to some extent with the test strains. The earlier studies of Futrelle[64] and of Marino[65] revealed the marked influence of cell concentration upon oxygen uptake, particularly so at reduced oxygen tension. This effect is shown in table 1, which indicates also that the actual percentage of respiratory inhibition is dependent in considerable part upon the concentration of cells employed. This was maintained constant for each pair of organisms studied in any one test, the pairing being done on a random basis.

If the results with the H37 strains are excluded—although these show the same pattern of inhibition as other pairs, particularly at the lowest concentration of cells employed (table 1)—the ranges are 29 to 44% for the inhibition of endogenous respiration of the virulent strains and 52% for the two avirulent ones. Respective figures for inhibition in the presence of lactate are 36–72 and 63–78; in Dubos-Davis medium 35–53 and 59–66; in serum 45–69 and 58–74; and in tissue juice 40–61 and 44–70. Quantitatively these figures leave much to be desired, but they create the over-all impression that in suspensions of the same turbidity prepared from cultures of the same age there is at least a marked tendency for the respiration of avirulent strains to be inhibited to a greater extent than that of virulent ones at 1% oxygen tension. On the basis of these considerations it was thought that a study of the growth of virulent and avirulent bacilli might reveal a differential influence of low oxygen tension more susceptible to quantitative study as well as more directly pertinent to the question of virulence mechanisms. The results of such a study are detailed in the following paper.

CONCLUSIONS

The evidence presented in this study suggests that avirulent strains of the tubercle bacillus generally respire in air at a more rapid rate than do their counterpart virulent strains of the same culture age adjusted to the same suspension turbidity. This relationship is maintained at 1% oxygen tension, but, in addition, respiration of the avirulent strains tends to be inhibited to a greater extent than that of the virulent strains. In many instances this difference in extent of inhibition is quite marked and appears to be independent of the nature of the suspending medium. Such a differential inhibition of respiration at reduced oxygen tensions might lead to a decreased rate of growth of avirulent strains in vitro and might possibly, in part at least, account for the failure of avirulent strains to multiply in the tissues of a host in which a virulent strain can multiply.

VIRULENCE OF THE TUBERCLE BACILLUS

II. EFFECT OF OXYGEN TENSION UPON GROWTH OF VIRULENT AND AVIRULENT BACILLI

L. RUTH GUY,* SIDNEY RAFFEL, AND C. E. CLIFTON

In the preceding paper[1] the current status of information concerning the mechanism of virulence of the tubercle bacillus was outlined, together with a rationale for the investigation of the possible relationship of oxygen tension of the environment to the fates of virulent, attenuated and avirulent bacilli in the tissues. A study by Warburg manometry of the respiratory activities of these bacilli at an oxygen tension of 1% revealed that this low concentration of oxygen results in inhibition of respiration of all organisms, but that the avirulent bacillus is apparently more seriously hindered in this respect than is the virulent. This, in turn, suggested that the factor of oxygen tension might be of importance in determining the inhibition of growth of avirulent bacilli in the body while virulent bacilli multiply progressively. In order to test this point directly the present experiments on growth were undertaken with a number of strains of human and bovine varieties of *Mycobacterium tuberculosis.*

MATERIALS AND METHODS

Eight strains of *Myco. tuberculosis* described in the preceding paper (H37Rv, H37Ra, Campbell, Cutter, Ravenel, BCG, JH6Ra, and JH16Ra),[1] two additional laboratory strains, H4Ra and 88, and five strains freshly isolated from patients were included in this study. Strain 88, a human virulent strain, was acquired in 1952 from Dr. C. Richard Smith of the Barlow Sanatorium, Los Angeles, California. Strain H4Ra, an avirulent human type variant, was obtained in 1950 from Mr. W. Steenken, Jr., of the Trudeau laboratory. The five latter cultures, Chris, Mel, Mose, Con, and Val, were recovered through, the kind cooperation of Drs. Lincoln Putnam and William Van Deventer, from the sputa of tuberculous patients at the Hassler Health Home, Redwood City, California. After proper treatment, the sputum samples were streaked on Dubos oleic acid-albumin medium containing penicillin, and transplants from isolated colonies were made to glycerin egg slants. No attempt was made to type these isolates.

The stock cultures were maintained on glycerin egg medium and transfers were made from them to Dubos Tween medium before the start of the growth experiments. The bacteria were subcultured in this liquid medium at intervals of 4 days in order to maintain the organisms in a state of rapid multiplication. This medium was employed because tubercle bacilli grow readily in it, clumping is not pronounced, and growth tends to be diffuse. Acid-fast stained smears of the stock cultures and of many of the test cultures were examined to detect possible contaminants. Flasks of uninoculated medium were incubated as sterility controls.

Four-day-old Tween medium cultures served as the inoculums in all experiments. These were diluted with fresh medium to a turbidity reading of 60 to 70 as determined by a Klett-Summerson photoelectric colorimeter equipped with a no. 54 green filter. In two experiments somewhat lighter suspensions were employed as the parent cultures, and in one test heavier inoculums were used. For inoculation, 10 ml amounts of the diluted bacterial suspensions were added to 200 ml of fresh medium, which was then dispensed in 15 ml amounts to sterile 50 ml cotton-stoppered Erlenmeyer flasks. The cultures were incubated at 37 C in a constant temperature room; one series was exposed to the air in the room, and the other was placed in 10 L glass desiccator jars

Received for publication August 8, 1953.

This work has been supported by grants from the National Tuberculosis Association, the California Tuberculosis and Health Association, and the Alameda County (California) Tuberculosis and Health Association.

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1. Heplar, J. Q., Clifton, C. E., Raffel, S. and Futrelle, C. M. 1953, J. Infect. Dis. **94**: 90.

equipped with inlet and outlet tubes for the passage of gas mixtures of reduced oxygen content. The gas mixture bubbled through a water bottle before entering the desiccator jar, flowed through the jar, and passed through another water bottle before discharging into the room. This second water bottle served as a barrier to prevent room air from entering through the outlet tube. A positive gas pressure was maintained in the desiccator jars at all times, with the gas passing into the chambers at a rate of about 60 bubbles per minute. After the jars were opened for the removal of sample flasks, the rate of flow of the gas mixture was accelerated for 15 minutes to obtain replacement of the air in the chambers. In all experiments oxygen passed by diffusion into the culture flasks; no gas was bubbled directly through them. Commercially prepared gas mixtures were employed for the growth experiments at lowered oxygen tension, 1% oxygen being employed with either 7.5 or 0.3% carbon dioxide and the remainder nitrogen. Experiments were also run using a mixture consisting of 92.47% nitrogen, 7.5% carbon dioxide, and 0.03% oxygen.

Sample flasks were removed at weekly intervals for determinations of amounts of growth. One experiment extended over 4 weeks, 7 over a period of 3 weeks, the remainder over 2 weeks. The amount of growth in each sample was determined by dry weight measurements. In a few experiments photographs were made of the cultures, after transfer to tubes for convenience of visualization, and of sediments deposited in hematocrit tubes by centrifugation.

When the sample cultures were removed from the incubator, any decrease in volume through evaporation was made up with distilled water. The flasks were then shaken, and a 1 ml sample of the culture was placed in a weighed glass vial. A second aliquot was centrifuged at 2000 r.p.m. for 20 minutes, and a 1 ml sample of the supernatant fluid was transferred to a second weighed vial. The vials containing culture or medium were placed in a drying oven maintained at 95 C and held at this temperature for 24 hours. After removal from the oven, the vials were allowed to stand at room temperature for 15 minutes and were then weighed to the nearest 0.1 mg. The weight of the dried supernatant medium was subtracted from the weight of the respective dried culture to obtain the dry weight of the organisms. Samples from other flasks containing the same original subculture were used for subsequent determinations of growth. An error might be introduced here if the organisms were not well dispersed in the originally seeded medium, but pains were taken to insure as equitable a distribution of bacilli as possible in all flasks at the initiation of the experiment.

RESULTS

Effect of 1% oxygen and 7.5% carbon dioxide on growth.—A series of experiments were performed in which parallel cultures of virulent, attenuated and avirulent bacilli of the same original turbidities were incubated in air and in a gaseous environment containing 1% oxygen and 7.5% carbon dioxide. The results obtained with 10 virulent strains, 4 avirulent variants, and 1 attenuated strain are summarized in table 1. From these data it is apparent that although the growth of all organisms was inhibited under the lowered oxygen tension, that of the avirulent strains was most markedly repressed. The single attenuated strain studied, BCG, resembled the virulent organisms more closely than the avirulent in its growth under reduced oxygen tension.

In figure 1 are presented photographs of cultures from experiments 12 and 13 after 3 weeks of growth. The differences in amounts of growth between the virulent and avirulent strains at low oxygen tension are plainly visible here. Differentials of this order could be seen on casual observation of most of the cultures in the various experiments reported. Figure 2 shows the packed cell sediments of 5 ml amounts of 2 virulent and 1 avirulent culture from the same experiments. In contrast, figure 3 portrays the similarity of growth of the attenuated strain BCG at 1% oxygen tension to that of the virulent bovine strain Ravenel with which it was compared.

In figure 4 the average growths of virulent and avirulent strains at low oxygen tension are presented as percentages of their respective growths in air in successive weeks. For the virulent

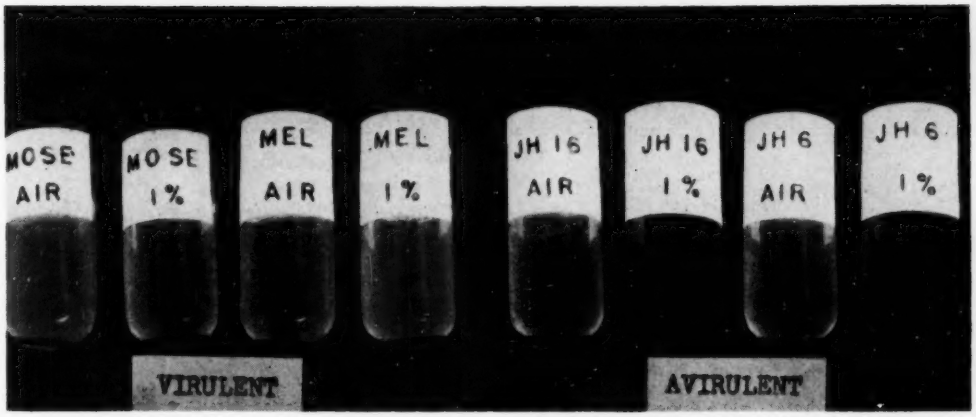

FIG. 1.—Three-week cultures of virulent and avirulent tubercle bacilli in air and in 1% oxygen.

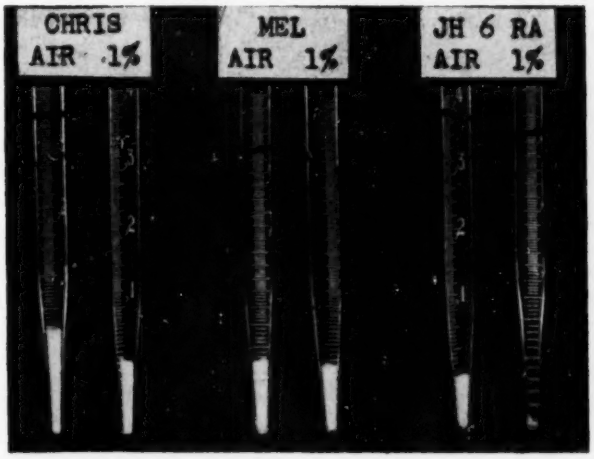

FIG. 2.—Centrifuged cells from 5 ml of cultures of virulent and avirulent tubercle bacilli after 3 weeks of growth in air and in 1% oxygen.

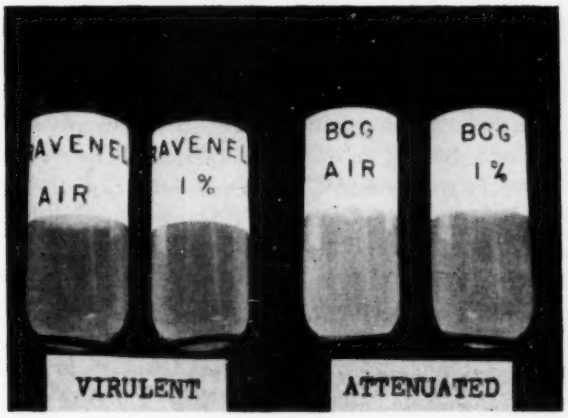

FIG. 3.—Three-week cultures of virulent (Ravenel) and attenuated (BCG) bovine strains in air and in 1% oxygen.

TABLE 1.—*Growth of* Myco. tuberculosis *in Dubos-Tween medium after 2 or 3 weeks' exposure to air or to 1% oxygen with 7.5 or 0.30% carbon dioxide.*

Exp. no.	Weeks	Strain	Inoculum O.D.*	Air Dry wt.†	1% O_2, 7.5% CO_2 Dry wt.†	1% O_2, 0.3% CO_2 Dry wt.†
1	2	H37Rv (V)	25	4.6	2.5	
		H37Ra (AV)	25	3.7	0.5	
2	3	H37Rv (V)	67	2.1	1.1	
		H37Ra (AV)	67	1.3	0.3	
3	2	H37Rv (V)	70	2.2	0.9	
		H37Ra (AV)	70	1.6	0.3	
		JH16Ra (AV)	70	1.8	0.2	
4	2	Campbell (V)	65	2.6	1.5	
		Cutter (V)	65	2.1	0.9	
		Ravenel (V)	65	2.6	0.9	
		BCG (AT)	65	2.1	1.2	
		H4Ra (AV)	65	1.9	0.3	
		JH16Ra (AV)	65	1.6	0.4	
5	2	H37Rv (V)	80	1.8	1.7	0.8
		H37Ra (AV)	80	1.7	0.6	0.4
6	2	H37Rv (V)	32	1.9	0.9	
		Ravenel (V)	32	1.8	0.4	
		BCG (AT)	32	1.9	0.5	
8	3	Campbell (V)	195	1.8	1.3	1.3
		H4Ra (AV)	195	1.3	0.5	0.4
9	3	H37Rv (V)	18	1.3	0.7	0.4
		H37Ra (AV)	18	1.0	0.3	0.1
		H4Ra (AV)	20	1.3	0.3	<0.1
10	2	88 (V)	52	1.1	0.1	0.1
		JH6Ra (AV)	54	0.8	0.1	0.1
11	3	Cutter (V)	65	2.3	1.0	0.8
		Val (V)	65	1.4	0.5	0.5
		JH6Ra (AV)	65	1.4	0.1	0.1
12	3	88 (V)	50	1.2	0.4	
		Val (V)	52	1.2	0.5	0.5
		Chris (V)	50	1.1	0.7	
		Ravenel (V)	50	1.3	0.8	0.6
		BCG (AT)	51	1.4	0.3	0.4
		JH6Ra (AV)	50	1.2	0.1	0.1
13	3	Chris (V)	65	1.2	1.1	
		Con (V)	65	1.6	0.7	
		Mel (V)	65	0.9	0.8	
		Mose (V)	65	1.3	0.7	
		Ravenel (V)	65	1.3	0.8	
		BCG (AT)	65	1.5	0.8	
		JH6Ra (AV)	65	1.3	0.2	
		JH16Ra (AV)	65	1.2	0.2	

V = virulent, AV = avirulent, AT = attenuated.
* O.D. = reading in Klett-Summerson colorimeter. O.D. of inoculum is that of 10 ml of the diluted parent culture which was added to 200 ml of medium for distribution to the experimental flasks.
† Dry weight of cells per ml of culture. The dry weight of the inoculum was generally in the range of 0.3 to 0.6 mg per ml of the diluted parent culture.

strains of all experiments the average growth (determined by dry weights) at the end of 1 week in an atmosphere containing 1% oxygen was 39.8% of the growth in air, at 2 weeks it was 45.4%; and at 3 weeks it was 56.2%. In contrast, corresponding figures for the avirulent variants were 22.5, 20.1 and 20.3%, respectively. The majority of cultures did not deviate markedly from these values. An exception was strain 88, a human virulent form which has pathogenicity for guinea pigs and grows in serpentine formation, but appears to resemble avirulent organisms in being markedly inhibited in its growth at low oxygen tension. The freshly isolated strain Val seems to be intermediate in its behavior. Both organisms are being investigated more thoroughly.

Effect of 1% oxygen and 0.30% carbon dioxide on growth.—A second gas mixture containing 1% oxygen with 0.30% carbon dioxide was employed in a number of growth experiments. This relatively low concentration of carbon dioxide was intended to reveal how far the effects ascribed to low oxygen tension above might be influenced by the accompanying carbon dioxide. These observations are also summarized in table 1. The differential of growth inhibition between virulent and avirulent strains is similar to that seen in the presence of the higher carbon dioxide tension. However, the growths of all organisms tended to be suppressed to a somewhat greater extent in the mixture containing the lower level of carbon dioxide. Several investigators have found that carbon dioxide in an amount between 2.5%[2] and the higher concentration of 7.5% used in the present work is somewhat stimulatory to the growth of human and bovine varieties of bacilli.[3,4] Although these various experiments were carried out in the presence of oxygen at or near atmospheric levels, in contrast to the presently reported work, some influence of carbon dioxide upon growth would seem to be corroborated by our results.

Effect of anaerobiasis upon viability of bacilli.—During the course of these experiments some cultures were exposed to a flowing mixture of 92.47% nitrogen, 7.5% carbon dioxide and 0.03% oxygen (present as an impurity in the tank mix-

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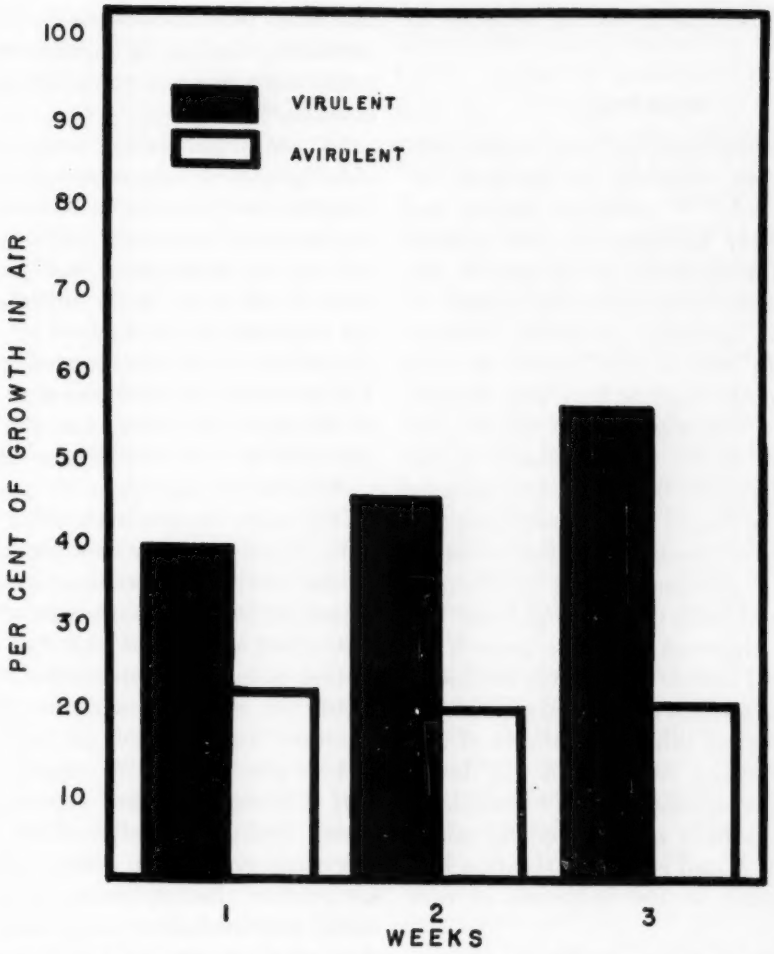

FIG. 4.—Growth in successive weeks of virulent and avirulent bacilli under 1% oxygen tension, expressed as percentage of growth in air.

ture). The purpose of this almost oxygen-free mixture was to assess the possible growth-promoting influence of carbon dioxide alone at the 7.5% concentration in the results seen in the mixture of this gas with 1% oxygen. As was expected in an environment practically devoid of oxygen, virulent, avirulent and attenuated bacilli all failed to show visible growth after 6 weeks of incubation. When the flasks were subsequently exposed to air for 3 weeks, no growth became evident. If the exposure to air was made after only 3 weeks of incubation in the oxygen-deficient atmosphere, however, growth appeared upon exposure to air. The unfavorable effects of anaerobiasis or very low concentrations of oxygen upon survival of tubercle bacilli has been known for some time,[3, 5–7] and recently Dubos[8] has also found that the presence of carbon dioxide and metabolites, such as sodium lactate and alanine, hasten

5. Novy, F. G. and Soule, M. H. 1925, J. Infect. Dis. **36**: 168.
6. Loebel, R. O., Shorr, E. and Richardson, H. B. 1933, J. Bact. **26**: 139, 167.
7. Potter, T. S. 1942, J. Infect. Dis. **71**: 220.
8. Dubos, R. J. 1953, J. Exper. Med. **97**: 357.

death of the bacilli in the absence of oxygen.

DISCUSSION

The results reported here reveal that, as has been observed by previous investigators,[3,5,9–11] tubercle bacilli are progressively hindered in their growth as the concentration of oxygen in the environment diminishes, regardless of the total amount available. Webb, Boissevain and Ryder[9] failed to find growth of the human bacillary variant even at 4 volumes percent of oxygen. Novy and Soule[5] found growth of the H37 strain under 0.5% concentration to be less than that in a flask containing 3% oxygen, even though the total amounts of oxygen consumed were practically the same in both cases. Corper and coworkers[3] observed that the growth of human and bovine strains was inhibited at 0.5% of oxygen in an open system.

The possible inhibitory effects of decreased oxygen tensions in the body upon the progression of the tuberculous process has been appreciated for some time,[3,5,6,11–17] and recently attention has turned again to the influence of this and other physicochemical factors in determining the fate of virulent bacilli in pulmonary lesions, especially in closed cavities.[18–22]

In the experiments recorded here, the 1% level of oxygen supplied in continuous flow to cultures of virulent, attenuated and avirulent bacilli of human and bovine types permitted growth to occur in all cases, in the modified manner expected on the basis of the reports of previous observers referred to above. The pertinent observation with respect to virulence, however, was the finding that this effect of low oxygen tension is a differential one, i.e., at a tension which may approximate that within cells, avirulent organisms are inhibited in their multiplication to a significantly greater extent than are virulent ones. This effect was noted with the concentration of oxygen-employed in these experiments with or without the concomitant presence of an amount of carbon dioxide (7.5%) approximating that of alveolar air and venous blood.[23] These findings parallel those of the preceding report,[1] in which it was observed that the respiration of avirulent bacilli was inhibited to a greater extent than that of virulent bacilli under reduced oxygen tension.

It is presently impossible to know exactly to what concentrations of oxygen tubercle bacilli within body cells may be exposed. Mixed venous blood

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14. Kempner, W. 1939, Am. Rev. Tuberc. **40**: 157.
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18. Coryllos, P. N. and Birnbaum, G. L. 1930, Arch. Surg. **21**: 1214.
19. Medlar, E. M. 1952, Am. Rev. Tuberc. **66**: 381.
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21. Falk, A., Hall, W., Tucker, W. B. and Kaufman, J. 1953, Abstr. 49th Ann. Meeting Natl. Tuberc. Assoc., p. 6.
22. Dubos, R. J. 1953, J. Exper. Med **97**: 357.
23. Best, C. H. and Taylor, N. B. 1950, The Physiological Basis of Medical Practice, ed. 5, Baltimore, Williams and Wilkins.

of man has an oxygen content of 12 to 14 volumes percent,[23] but the oxygen concentration in an area of inflammation may be as low as 0.8 percent.[24] Calculating on the basis of the absorption coefficient of oxygen in plasma at body temperature, the 1% oxygen atmosphere to which the liquid cultures in these experiments were exposed should have resulted in a concentration in the medium of 0.023 volumes percent of oxygen. This figure is in the range of actual measurements of dissolved oxygen in various culture mediums made by Knaysi and Dutky.[25,11] Loebel and co-workers[6] have computed that complete anaerobiasis may obtain in the center of a tubercle with a radius of only 0.075 mm. Undoubtedly the tubercle bacillus in its intracellular habitat is exposed to very low levels of oxygen, possibly in the neighborhood of that which our experimental conditions imposed. It might be inferred that the concentrations of oxygen available to organisms in the body are actually somewhat less than this, at a level where the differential effect could be more pronounced, virulent organisms being further inhibited than in these experiments while avirulent bacilli are completely suppressed.

Although it has been observed by others[26,27] as well as by ourselves that avirulent organisms generally grow less well under ordinary atmospheric conditions than do virulent ones, the results reported here are not contingent upon this fact, for it is the proportionately greater decrease of multiplication of avirulent bacilli at the low oxygen tension over their growth in air which is of significance. As a matter of fact, the H37Ra strain has been found by Holmgren and Youmans[28] to have the same generation time as the virulent strain H37Rv. As shown in table 1, in certain of the comparative experiments reported here this avirulent organism grew more profusely than its companion H37Rv, yet the avirulent strain showed appreciably less growth at the low oxygen concentration than did the virulent. Bearing upon this point also is the fact that the freshly isolated virulent strains with which we worked did not grow as rapidly in air as the laboratory strains; rather, they resembled the avirulent variants in this respect. Yet here again the differential effect with respect to growth at low oxygen tension was apparent.

It is interesting that in the four experiments in which the attenuated strain, BCG, was studied, its growth behavior was more characteristic of the virulent than of the avirulent strains. This coincides with other observed traits of this bacterium, including its colonial properties of spreading and serpentine growth, its reaction to the neutral red test,[26,29] and its ability to propagate within macrophages in tissue cultures.[30] Furthermore, it appears to multiply to some extent in the tissues, in contrast to the nondeveloping avirulent organisms.[31–35] The studies of

24. Kempner, W. 1930, Ztschr. f. klin. Med. **114:** 439.
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27. Steenken, W., Jr. Personal communication to Middlebrook.[26]
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30. Suter, E. 1952, J. Exper. Med. **96:** 137.
31. Griffith, A. S. 1931, Gr. Brit. Med. Res. Counc. Spec. Rep. Series #152.
32. Lurie, M. B. 1934, J. Exper. Med. **60:** 163.
33. Birkhaug, K. E. 1933, Am. Rev. Tuberc. **27:** 6.
34. Stewart, G. T. and Tamargo-Sanchez, M. 1952, J. Hyg. **50:** 37.
35. Ratcliffe, H. L. and Palladino, V. S. 1953, J. Exper. Med. **97:** 61.

Lurie[32] and of Pierce and co-workers[36] imply that the failure of these bacteria to cause progressive infection in mice may owe to an acquired property of the host, for the bacillus multiplies for a time after injection and then decreases in numbers. In contrast, avirulent bacilli fail to propagate at all, while virulent organisms continue to multiply to the death of the host.

CONCLUSIONS

Fifteen strains of virulent, attenuated and avirulent tubercle bacilli of human and bovine types in Dubos-Davis medium were subjected in an open system to atmospheres containing 1% oxygen with or without 7.5% carbon dioxide. As compared directly to identical cultures cultivated in air, all strains were inhibited in their growth. Multiplication of the avirulent organisms, however, was more markedly suppressed than that of the virulent strains.

The concentration of carbon dioxide exerted little influence upon the course of growth in any of these instances studied. Growth of all strains tended to be suppressed more at a lower (0.30%) than a higher (7.5%) concentration of carbon dioxide in the atmosphere of incubation.

There is evidence that an attenuated bacillus, BCG, displays the growth behavior of virulent rather than of avirulent organisms under low oxygen tension. This behavior coincides with other activities of this bacterium.

It seems probable from these observations that a major factor determining the virulence of the tubercle bacillus may be its ability to proliferate in an environment deficient in oxygen. The avirulent organism, in contrast, is much less able to utilize low concentrations of oxygen for its multiplication.

36. Pierce, C., Dubos, R. J. and Schaefer, W.B. 1953, J. Exper. Med. **97**: 189.